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(71) Applicant *(for all designated States except US)*: UNIVERSITY OF HAWAII [US/US]; Suite 280, 2800 Woodlawn Drive, Honolulu, HI 96822 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: NAN, Guo-Ling [-/US]; 775 Kinalau Place #1207, Honolulu, HI 96813 (US). NAGAI, Chifumi [JP/US]; 1021 Lunaai Street, Kailua, HI 96734 (US).

(74) Agents: CARROLL, Peter, G. et al.; Medlen & Carroll, LLP, Suite 2200, 220 Montgomery Street, San Francisco, CA 94104 (US).

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(54) Title: COMPOSITIONS AND METHODS FOR GENETIC TRANSFORMATION OF PINEAPPLE

(57) Abstract

The present invention provides methods for the production of transgenic pineapple-like totipotent bodies, and in particular, transgenic pineapple-like callus and transgenic pineapple-like protocorm-like bodies. Also provided by this invention are methods for the production of transgenic plants from transgenic totipotent bodies which include transgenic pineapple-like callus and protocorm-like bodies. The invention additionally provides transgenic pineapple-like plants which may be genetically engineered to exhibit resistance to pests and disease and to exhibit improved qualities. The invention further provides improved methods for the maintenance of pineapple-like protocorm-like bodies in culture. These improved methods are useful for reducing the time, cost, and labor involved in selecting stably transformed pineapple-like protocorm-like bodies.

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COMPOSITIONS AND METHODS FOR GENETIC TRANSFORMATION OF PINEAPPLE

This invention was made with Government support under ARS Agreement No.: 59-5320-5-693 awarded by the United States Department of Agriculture. The Government has certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to improved methods for *in vitro* culture of pineapple-like protocorm-like bodies and to methods for the production of transgenic pineapple-like plants. In particular, this invention relates to the *in vitro* culture of pineapple-like protocorm like bodies, and to the genetic transformation of pineapple-like protocorm-like bodies and of pineapple-like callus to produce transgenic pineapple-like plants.

BACKGROUND OF THE INVENTION

Bromeliads (*i.e.*, the family Bromeliaceae) are a family of plants which includes a wide variety of ornamental plants as well as plants valued for their edible fruit. Foremost among the members of the Bromeliaceae family is pineapple which bears a fruit that is valuable both to the commercial producer and to the consumer, both of whom have come to expect certain characteristic in the fruit produced and consumed. For example, while characteristics which are valued by the commercial producer include pest and disease resistance of both the plant and fruit, a high yield of fruit, high brix, and extended shelf-life, those valued by the consumer include low acidity (particularly in winter fruit), low fiber, and uniform fruit shape and color.

Efforts in the pineapple production industry geared to meeting these demands have focuses on agroniomic management of crops in order to generate fresh fruit with a total soluble solids (TSS) in the range of 15-20%, titratable acidity of 10.0 to 12.0 with minimal summer to winter variation, and high ascorbic acid (vitamin C) for reduced chilling injury symptom development. Traditionally, meeting these demands

has focused on vegetative propagation as well as on breeding of pineapple plants with the desired characteristics. However, extensive breeding efforts have produced only a limited number of new varieties with desirable characteristics. Similarly, while traditional vegetative propagation [*e.g.*., by means of lateral shoots, basal sucker, or crowns (see, *e.g.*, Collins *et al.* (1938) J. Heredity 29:163-164)] has been used to propagate pineapple commercially, the limited availability of plant material for vegetative propagation, as well as the slow multiplication rate of vegetatively propagated tissue have prompted the use of an *in vitro* propagation approach in order to meet the growing worldwide demand for pineapple fruit.

While *in vitro* culture methods for the generation of pineapple plants are available [Dewald *et al* (1988) Plant Cell Rep. 7:535-537; Drew *et al.* (1980) Queesnsland Agr. J. 106:447-451; Firoozabady *et al.* (1995) In Vitro Cell. Develop. Biol. 31:51A; Hirimburegama *et al.* (1992) Acta Hort. 319:203-208; Kiss *et al.* (1995) HortSci 30:127-129; Mapes (1973) Proc. Intl. Plant Propagation Soc. 23:47-55; Mathews *et al.* (1979) Scientia Horticulturae 11:319-328; Mathews *et al.* (1981) Scientia Hort. 14:227-234; Wakasa *et al.* (1978) Japan. J. Breed. 28:113-121; Wakasa (1979) Japan. J. Breed. 29:13-22], attempts to use pineapple tissue culture for the propagation of plants with desirable characteristics have also been unsatisfactory due to the variability in regenerated plants.

What is needed is a better method for producing pineapple plants with desirable characteristics. This method should be amenable to manipulation such that desirable characteristics may be selectively introduced into a pineapple plant which may subsequently be used for the further propagation of pineapple plants with the same characteristics.

SUMMARY OF THE INVENTION

In one embodiment, the present invention provides a stably transformed transgenic pineapple-like totipotent body. While not intending to limit the invention to a particular totipotent body, in a preferred embodiment, the totipotent body is a

protocorm-like body. In an alternative preferred embodiment, the totipotent body is a callus. In yet another embodiment, the totipotent body comprises undifferentiated cells. In a further preferred embodiment, the pineapple-like totipotent body is a pineapple totipotent body.

In another embodiment, the invention provides a stably transformed transgenic pineapple-like plant. Without limiting the type of tissue from which the transgenic pineapple-like plant is derived, in one preferred embodiment, the plant is derived from a transgenic pineapple-like totipotent body. In a further preferred embodiment, the totipotent body is a protocorm-like body. In yet a further preferred embodiment, the totipotent body is a callus. Without intending to limit the type of plant, in one preferred embodiment, the plant is a pineapple plant.

Yet another embodiment of the present invention is a method for producing a stably transformed transgenic pineapple-like totipotent body, comprising: a) providing: i) a pineapple-like totipotent body; and ii) a heterologous nucleic acid comprising an oligonucleotide sequence of interest; and b) introducing the oligonucleotide sequence of interest into the totipotent body under conditions such that a stably transformed transgenic pineapple-like totipotent body is produced. While not intending to limit the type of totipotent body, in one preferred embodiment, the totipotent body is a callus. In another preferred embodiment, the totipotent body is a protocorm-like body. In another embodiment, the method further comprises c) selecting the transgenic totipotent body. In a preferred embodiment, the selecting is in liquid medium. In an alternative preferred embodiment, the selecting comprises detecting the oligonucleotide in the genome of the totipotent body. In yet another embodiment, the pineapple-like totipotent body is a pineapple totipotent body. In yet a further embodiment, the oligonucleotide is introduced by bombarding the totipotent body with the oligonucleotide sequence of interest. In an alternative preferred embodiment, the oligonucleotide is introduced by infecting the totipotent body with *Agrobacterium* comprising the oligonucleotide sequence of interest. Without intending to limit the method of infection, in one preferred embodiment, the infecting comprises microwounding the totipotent body to produce a microwounded totipotent body, and

infecting the microwounded totipotent body with the *Agrobacterium*. In a further preferred embodiment, the *Agrobacterium* is agropine-type. In an alternative preferred embodiment, the *Agrobacterium* is nopaline-type. In yet another alternative preferred embodiment, the *Agrobacterium* is octopine-type.

In one embodiment, the present invention provides a method for producing a stably transformed transgenic pineapple-like plant, comprising: a) providing: i) a pineapple-like totipotent body; and ii) a heterologous nucleic acid comprising an oligonucleotide sequence of interest; b) introducing the oligonucleotide sequence of interest into the totipotent body under conditions such that a transgenic pineapple-like totipotent body is produced; and c) culturing the transgenic pineapple-like totipotent body under conditions such that a stably transformed transgenic pineapple-like plant is produced. Without intending to limit the invention to a particular method of introducing the oligonucleotide, in one preferred embodiment, the oligonucleotide is introduced by bombarding the pineapple-like totipotent body with the oligonucleotide sequence of interest. In an alternative preferred embodiment, the oligonucleotide is introduced by infecting the pineapple-like totipotent body with *Agrobacterium* comprising the oligonucleotide sequence of interest. In yet another alternative embodiment, the method further comprises prior to step c) selecting the transgenic totipotent body. In a particularly preferred embodiment, the selecting is in liquid medium. In a preferred embodiment, the selecting comprises detecting the oligonucleotide in the genome of the pineapple-like totipotent body. In one embodiment, the pineapple-like plant is a pineapple plant. In an alternative embodiment, the sequence of interest is selected from the group consisting of oligonucleotides encoding sucrose phosphate synthase, CpTi, thaumatin, and ACC deaminase. In yet another alternative embodiment, the sequence of interest is selected from the group consisting of antisense polyphenol oxidase and ACC oxidase.

In another embodiment, the invention provides a method for producing a pineapple-like protocorm-like body comprising maintaining the pineapple-like protocorm-like body in liquid medium. Without intending to restrict the type of

medium to a particular composition, in one embodiment, the liquid medium substantially comprises PI medium. In an alternative embodiment, the pineapple-like plant is a pineapple plant.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 schematically shows the structure of pBI426.

Figure 2 schematically shows the structure of pBI121.

Figure 3 schematically shows the nucleotide sequence of the 5' transcribed region of the rice *Act*1-*Gus* gene fusion in p*Act1*-F between the *Act1* TATA box and *Gus* translation initiation codon.

Figure 4 schematically shows the structure pAHC27.

Figure 5 schematically shows the structure of pHA9.

Figure 6 schematically shows the structure of pEmuGN.

Figure 7 schematically shows the structure of pWD1.

Figure 8 schematically shows the structure of pWD3.

Figure 9 schematically shows the structure of pML72.

Figure 10 schematically shows the structure of pML75.

Figure 11 shows transient expression of GUS protein as measured by histochemical staining in pineapple (A) leaf, (B) lead and fruit, and (C) flower petal used as target tissue for biolistic bombardment of expression constructs containing the *Gus* gene.

Figure 12 shows an antibiotic growth inhibition curve for F153 (A and C) and for D10 (B and D) protocorm-like bodies (plbs) treated for one month (open circles) or two months (filled squares) with different G418 concentrations in liquid PI media (A and B) and agar-solidified shoot regeneration PM2 media (C and D).

Figure 13 shows the nucleotide sequence (SEQ ID NO:1) encoding polyphenol oxidase.

Figure 14 shows the nucleotide sequence (SEQ ID NO:2) encoding maize sucrose phosphate synthase enzyme.

Figure 15 shows a Southern hybridization blot of genomic DNA isolated from pineapple leaves transfected with pBI426 and PBI121 using biolistic bombardment.

DEFINITIONS

The term "totipotent body" as used herein refers to a collection of cells (*e.g.*, a cell aggregate) comprising undifferentiated plant cells capable of differentiation into a plant; an totipotent body may also contain some differentiated cells. An "totipotent body" includes, but is not limited to, a protocorm-like body, a callus, and the like. The ability of an totipotent body to differentiate into a plant is determined using methods known in the art as well as methods described herein. For example, differentiation into shoots may be accomplished by culturing an totipotent body on agar-solidified hormone-free modified MS medium, or on agar-solidified PM2 medium. Differentiation into roots may be accomplished by culture of an totipotent body in liquid modified MS medium containing 1 mg/L NAA.

The terms "protocorm-like body," "plb," and "nodular body" when made in reference to pineapple refer to an totipotent body which is generally, though not necessarily, creamy yellow and globular shaped. A plb derived form pineapple tissue is morphologically distinguishable from a callus derived from pineapple. For example, a plb which is derived from pineapple tissue is characterized by having a partially organized morphology with a pre-determined apical meristematic region covered by a distinctive epidermal layer but lacking vascular tissue; a callus derived from pineapple tissue is a disorganized mass of undifferentiated plant cells lacking apical meristem, epidermis, and vascular tissue. Additionally, *in vitro* culture conditions for the generation of a plb are different from those for the generation of a callus. For example, pineapple plbs may be generated as previously described (Wakasa *et al.* (1978) Japan. J. Breed. 28:113-121; Mapes (1973) Proc. Intl. Plant Propagation Soc. 23:47-55) and using methods described herein. Pineapple callus may be produced as described by (Mathews *et al.* (1981) Scientia Horiculturae 14:227-234).

The term "plant" as used herein refers to a plurality of plant cells which are largely differentiated into a structure that is present at any stage of a plant's

development. Such structures include, but are not limited to, a fruit, shoot, stem, leaf, flower petal, *etc.*

The term "pineapple" refers to a member of the genera *Ananas* and *Pseudoananas* of the Bromeliaceae family. The genus *Pseudoananas* is monotypic, *i.e.*, consists of *P. sagenarius*. The genus *Ananas* consists of five species, namely, *A. bracteatus, A. fritzmuelleri, A. comosus, A. erectifolius,* and *A. ananassoides.*

The term "pineapple-like" refers to a member of the Bromeliaceae family. Genera comprised within the Bromeliaceae family include *Tillansia, Aechmea, Neoregrelia*, etc.

The term "PI medium" refers to supplemented modified MS medium. Modified MS medium refers to the medium described by Murashige & Skoog (Murashige & Skoog, (1962) Physiol. Plant 15::473-497) (Gibco BRL) which is modified by the addition of 100 mg/L myo-inositol (Sigma), 1 mg/L thiamine-HCl (Sigma), and 30 g sucrose (Sigma), pH 5.6-5.8, and supplemented by the addition either of 2 mg/L α-naphtaleneacetic acid (NAA) (Sigma) and 1 mg/L 6-benzyl aminopurine (BAP) (Sigma), or of 10 mg/L NAA and 1 mg/L BAP.

The term "transgenic" when used in reference to a protocorm-like body refers to a protocorm-like body which comprises one or more cells that contain a transgene, or whose genome has been altered by the introduction of a transgene. The term "transgenic" when used in reference to a plant refers to a plant which comprises one or more cells which contain a transgene, or whose genome has been altered by the introduction of a transgene. These transgenic plbs and transgenic plants may be produced by several methods including the introduction of a "transgene" comprising nucleic acid (usually DNA) into a target cell or integration into a chromosome of a target cell by way of human intervention, such as by the methods described herein.

The term "transgene" as used herein refers to any nucleic acid sequence which is introduced into the genome of a plant cell by experimental manipulations. A transgene may be an "endogenous DNA sequence," or a "heterologous DNA sequence" (*i.e.*, "foreign DNA"). The term "endogenous DNA sequence" refers to a nucleotide

sequence which is naturally found in the cell into which it is introduced so long as it does not contain some modification (*e.g.*, a point mutation, the presence of a selectable marker gene, etc.) relative to the naturally-occurring sequence. The term "heterologous DNA sequence" refers to a nucleotide sequence which is ligated to, or is manipulated to become ligated to, a nucleic acid sequence to which it is not ligated in nature, or to which it is ligated at a different location in nature. Heterologous DNA is not endogenous to the cell into which it is introduced, but has been obtained from another cell. Heterologous DNA also includes an endogenous DNA sequence which contains some modification. Generally, although not necessarily, heterologous DNA encodes RNA and proteins that are not normally produced by the cell into which it is expressed. Examples of heterologous DNA include reporter genes, transcriptional and translational regulatory sequences, selectable marker proteins (*e.g.*, proteins which confer drug resistance), *etc.*

The term "gene" refers to a DNA sequence that comprises control and coding sequences necessary for the production of a polypeptide or its precursor. The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence.

The term "transformation" as used herein refers to the introduction of foreign DNA into cells. Transformation of a plant cell may be accomplished by a variety of means known in the art including particle mediated gene transfer (see, *e.g.*, U.S. Patent No. 5,584,807 hereby incorporated by reference); infection with an *Agrobacterium* strain containing the foreign DNA for random integration (U.S. Patent No. 4,940,838 hereby incorporated by reference) or targeted integration (U.S. Patent No. 5,501,967 hereby incorporated by reference) of the foreign DNA into the plant cell genome; electroinjection (Nan *et al.* (1995) In "Biotechnology in Agriculture and Forestry," Ed. Y.P.S. Bajaj, Springer-Verlag Berlin Heidelberg, Vol 34:145-155; Griesbach (1992) HortScience 27:620); fusion with liposomes, lysosomes, cells, minicells or other fusible lipid-surfaced bodies (Fraley *et al.* (1982) Proc. Natl. Acad. Sci. USA 79:1859-

1863; polyethylene glycol (Krens *et al.* (1982) nature 296:72-74); chemicals that increase free DNA uptake; transformation using virus, and the like.

Transformation of a cell may be stable or transient. The term "transient transformation" or "transiently transformed" refers to the introduction of one or more transgenes into a cell in the absence of integration of the transgene into the host cell's genome. Transient transformation may be detected by, for example, enzyme-linked immunosorbent assay (ELISA) which detects the presence of a polypeptide encoded by one or more of the transgenes. The term "transient transformant" refers to a cell which has transiently integrated one or more transgenes. In contrast, the term "stable transformation" or "stably transformed" refers to the introduction and integration of one or more transgenes into the genome of a cell. Stable transformation of a cell may be detected by Southern blot hybridization of genomic DNA of the cell with nucleic acid sequences which are capable of binding to one or more of the transgenes. The term "stable transformant" refers to a cell which has stably integrated one or more transgenes into the genomic DNA. Thus, a stable transformant is distinguished from a transient transformant in that, whereas genomic DNA from the stable transformant contains one or more transgenes, genomic DNA from the transient transformant does not contain a transgene.

The term "nucleic acid sequence of interest" refers to any nucleic acid sequence the manipulation of which may be deemed desirable for any reason by one of ordinary skill in the art (*e.g.*, confer improved qualities).

The term "wild-type" when made in reference to a gene refers to a gene which has the characteristics of a gene isolated from a naturally occurring source. The term "wild-type" when made in reference to a gene product refers to a gene product which has the characteristics of a gene product isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designated the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" when made in reference to a gene or to a gene product refers, respectively, to a gene or to a gene product which displays modifications in sequence and or functional properties (*i.e.*, altered characteristics) when compared to

the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

The term "antisense" as used herein refers to a deoxyribonucleotide sequence whose sequence of deoxyribonucleotide residues is in reverse 5' to 3' orientation in relation to the sequence of deoxyribonucleotide residues in a sense strand of a DNA duplex. A "sense strand" of a DNA duplex refers to a strand in a DNA duplex which is transcribed by a cell in its natural state into a "sense mRNA." Thus an "antisense" sequence is a sequence having the same sequence as the non-coding strand in a DNA duplex. The term "antisense mRNA" refers to a ribonucleotide sequence whose sequence is complementary to an "antisense" sequence.

The term "recombinant" when made in reference to a DNA molecule refers to a DNA molecule which is comprised of segments of DNA joined together by means of molecular biological techniques. The term "recombinant" when made in reference to a protein or a polypeptide refers to a protein molecule which is expressed using a recombinant DNA molecule.

As used herein, the terms "vector" and "vehicle" are used interchangeably in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another.

The term "expression vector" or "expression cassette" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

The terms "targeting vector" or "targeting construct" refer to oligonucleotide sequences comprising a gene of interest flanked on either side by a recognition sequence which is capable of homologous recombination of the DNA sequence located between the flanking recognition sequences.

The terms "in operable combination", "in operable order" and "operably linked" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

The term "Southern blot" refers to the analysis of DNA on agarose or acrylamide gels to fractionate the DNA according to size, followed by transfer and immobilization of the DNA from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized DNA is then probed with a labeled oligo-deoxyribonucleotide probe or DNA probe to detect DNA species complementary to the probe used. The DNA may be cleaved with restriction enzymes prior to electrophoresis. Following electrophoresis, the DNA may be partially depurinated and denatured prior to or during transfer to the solid support. Southern blots are a standard tool of molecular biologists [J. Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, NY, pp 9.31-9.58].

The terms "infecting" and "infection" with a bacterium refer to co-incubation of a target biological sample, (*e.g.*, cell, tissue, *etc.*) with the bacterium under conditions such that nucleic acid sequences contained within the bacterium are introduced into one or more cells of the target biological sample.

The term "*Agrobacterium*" refers to a soil-borne, Gram-neggative, rod-shaped phytopathogenic bacterium which causes crown gall. The term "*Agrobacterium*" includes, but is not limited to, the strains *Agrobacterium tumefaciens*, (which typically causes crown gall in infected plants), and *Agrobacterium rhizogens* (which causes hairy root disease in infected host plants). Infection of a plant cell with *Agrobacterium* generally results in the production of opines (*e.g.*, nopaline, agropine, octopine etc.) by the infected cell. Thus, *Agrobacterium* strains which cause production of nopaline (*e.g.*, strain LBA4301, C58, A208) are referred to as "nopaline-type" *Agrobacteria*; *Agrobacterium* strains which cause production of octopine (*e.g.*,

strain LBA4404, Ach5, B6) are referred to as "octopine-type" *Agrobacteria*; and *Agrobacterium* strains which cause production of agropine (*e.g.*, strain EHA105, EHA101, A281) are referred to as "agropine-type" *Agrobacteria*.

The terms "bombarding, "bombardment," and "biolistic bombardment" refer to the process of accelerating particles towards a target biological sample (*e.g.*, cell, tissue, *etc.*) to effect wounding of the cell membrane of a cell in the target biological sample and/or entry of the particles into the target biological sample. Methods for biolistic bombardment are known in the art (*e.g.*, U.S. Patent No. 5,584,807, the contents of which are herein incorporated by reference), and are commercially available (*e.g.*, the helium gas-driven microprojectile accelerator (PDS-1000/He) (BioRad).

The term "microwounding" when made in reference to plant tissue refers to the introduction of microscopic wounds in that tissue. Microwounding may be achieved by, for example, particle bombardment as described herein.

As used herein, the term "probe" when made in reference to an oligonucleotide (*i.e.*, a sequence of nucleotides) refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, recombinantly or by PCR amplification, which is capable of hybridizing to another oligonucleotide of interest. A probe may be single-stranded or double-stranded. Probes are useful in the detection, identification and isolation of particular gene sequences. Oligonucleotide probes may be labelled with a "reporter molecule," so that the probe is detectable using a detection system. Detection systems include, but are not limited to, enzyme, fluorescent, radioactive, and luminescent systems.

The term "selectable marker" as used herein, refer to a gene which encodes an enzyme having an activity that confers resistance to an antibiotic or drug upon the cell in which the selectable marker is expressed. Selectable markers may be "positive" or "negative." Examples of positive selectable markers include the neomycin phosphotrasferase (NPTII) gene which confers resistance to G418 and to kanamycin, and the bacterial hygromycin phosphotransferase gene (*hyg*), which confers resistance

to the antibiotic hygromycin. Negative selectable markers encode an enzymatic activity whose expression is cytotoxic to the cell when grown in an appropriate selective medium. For example, the HSV-*tk* gene is commonly used as a negative selectable marker. Expression of the HSV-*tk* gene in cells grown in the presence of gancyclovir or acyclovir is cytotoxic; thus, growth of cells in selective medium containing gancyclovir or acyclovir selects against cells capable of expressing a functional HSV TK enzyme.

The terms "promoter element," "promoter," or "promoter sequence" as used herein, refer to a DNA sequence that is located at the 5' end (*i.e.* precedes) the protein coding region of a DNA polymer. The location of most promoters known in nature precedes the transcribed region. The promoter functions as a switch, activating the expression of a gene. If the gene is activated, it is said to be transcribed, or participating in transcription. Transcription involves the synthesis of mRNA from the gene. The promoter, therefore, serves as a transcriptional regulatory element and also provides a site for initiation of transcription of the gene into mRNA.

The term "amplification" is defined as the production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction technologies well known in the art [Dieffenbach CW and GS Dveksler (1995) *PCR Primer, a Laboratory Manual*, Cold Spring Harbor Press, Plainview NY]. As used herein, the term "polymerase chain reaction" ("PCR") refers to the method of K.B. Mullis disclosed in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,965,188, all of which are hereby incorporated by reference, which describe a method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their

complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension can be repeated many times (*i.e.*, denaturation, annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified."

With PCR, it is possible to amplify a single copy of a specific target sequence in genomic DNA to a level detectable by several different methodologies (*e.g.*, hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; and/or incorporation of ^{32}P-labeled deoxyribonucleotide triphosphates, such as dCTP or dATP, into the amplified segment). In addition to genomic DNA, any oligonucleotide sequence can be amplified with the appropriate set of primer molecules. In particular, the amplified segments created by the PCR process itself are, themselves, efficient templates for subsequent PCR amplifications. Amplified target sequences may be used to obtain segments of DNA (*e.g.*, genes) for the construction of targeting vectors, transgenes, etc.

The term "hybridization" as used herein refers to any process by which a strand of nucleic acid joins with a complementary strand through base pairing [Coombs J (1994) *Dictionary of Biotechnology*, Stockton Press, New York NY].

As used herein, the terms "complementary" or "complementarity" when used in reference to polynucleotides refer to polynucleotides which are related by the base-pairing rules. For example, for the sequence 5'-AGT-3' is complementary to the sequence 5'-ACT-3'. Complementarity may be "partial," in which only some of the

nucleic acids' bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods which depend upon binding between nucleic acids.

The term "homology" when used in relation to nucleic acids refers to a degree of complementarity. There may be partial homology or complete homology (*i.e.*, identity). A partially complementary sequence is one that at least partially inhibits a completely complementary sequence from hybridizing to a target nucleic acid is referred to using the functional term "substantially homologous." The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or Northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (*i.e.*, the hybridization) of a sequence which is completely homologous to a target under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (*i.e.*, selective) interaction. The absence of non-specific binding may be tested by the use of a second target which lacks even a partial degree of complementarity (*e.g.*, less than about 30% identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target.

Low stringency conditions when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l $NaH_2PO_4 \cdot H_2O$ and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.1% SDS, 5X Denhardt's reagent [50X Denhardt's contains per 500 ml: 5 g Ficoll (Type 400, Pharmacia), 5 g BSA (Fraction V; Sigma)] and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution

comprising 5X SSPE, 0.1% SDS at 42°C when a probe of about 500 nucleotides in length is employed.

High stringency conditions when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l $NaH_2PO_4 \cdot H_2O$ and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5X Denhardt's reagent and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 0.1X SSPE, 1.0% SDS at 42°C when a probe of about 500 nucleotides in length is employed.

When used in reference to nucleic acid hybridization the art knows well that numerous equivalent conditions may be employed to comprise either low or high stringency conditions; factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, etc.) and the concentration of the salts and other components (*e.g.*, the presence or absence of formamide, dextran sulfate, polyethylene glycol) are considered and the hybridization solution may be varied to generate conditions of either low or high stringency hybridization different from, but equivalent to, the above listed conditions.

"Stringency" when used in reference to nucleic acid hybridization typically occurs in a range from about T_m-5°C (5°C below the T_m of the probe) to about 20°C to 25°C below T_m. As will be understood by those of skill in the art, a stringent hybridization can be used to identify or detect identical polynucleotide sequences or to identify or detect similar or related polynucleotide sequences. Under "stringent conditions" a nucleic acid sequence of interest will hybridize to its exact complement and closely related sequences.

DESCRIPTION OF THE INVENTION

The present invention provides methods for the production of transgenic pineapple-like totipotent bodies. In particular, this invention provides methods for

producing transgenic pineapple-like callus and transgenic pineapple-like protocorm-like bodies.

Also provided by this invention are methods for the production of transgenic plants from transgenic totipotent bodies. More particularly, the invention discloses methods for generating transgenic pineapple-like plants from transgenic pineapple-like protocorm-like bodies and from transgenic pineapple-like callus. Yet more particularly, the present invention provides methods for generating transgenic pineapple-like plants from plbs and callus.

The invention additionally provides transgenic pineapple-like plants. The pineapple-like plants provided by this invention may be genetically engineered to exhibit resistance to pests and disease (*e.g.*, mealybugs, nematodes, root-rot and heart-rot disease, wilt virus, *etc.*) and to environmental stress (*e.g.*, water-logging, drought, heat, cold, light-intensity, day-length, chemicals, *etc.*) and to exhibit improved qualities (*e.g.*, longer storage life, higher yield, higher sugar content, higher vitamins C and A content, lower acidity, non-translucent flesh color, longer storage, *etc.*).

The invention further provides improved methods for the maintenance of pineapple-like protocorm-like bodies in culture. These methods are useful for the selection of stably transformed pineapple-like protocorm-like bodies. Such selection reduces the time, cost, and labor involved in screening for the presence of transgenic plants regenerated directly from protocorm-like bodies which are subjected to transformation protocols in the absence of a selection step to enrich for stably transformed protocorm-like bodies. These methods are also useful for the amplification of the number of transformed protocorm-like bodies, thus providing a larger amount of starting material for the regeneration of a greater number of cloned plants.

Without intending to restrict the scope of the present invention to a particular pineapple-like plant, the description of the invention is divided into (a) generation of pineapple totipotent bodies, (b) maintenance in *in vitro* culture of pineapple protocorm-

like bodies, (c) generation of transgenic pineapple totipotent bodies, and (d) generation of transgenic pineapple plants.

A. Generation of Pineapple Totipotent Bodies

Totipotent bodies derived from pineapple comprise protocorm-like bodies and callus. Pineapple protocorm-like bodies are generated by *in vitro* culture of pineapple explants from different tissues as previously described (Wakasa *et al.* (1978) *supra*; Mapes (1973), *supra*) as well as using methods described herein. For example, explants from the crown and vegetative shoots may be used to initiate plb cultures by placing the explants in media containing growth regulators.

The generation of plbs is not limited to explants from crown tissue or vegetative shoot tissue. Any pineapple tissue which is capable of generating plbs in *in vitro* culture is contemplated to be within the scope of this invention.

In one embodiment the media used to initiate plb formation is liquid modified MS medium containing a combination of NAA (α-naphtaleneacetic acid) and BAP (6-benzyl aminopurine). The liquid medium used for the initiation of plbs is not limited to the media or to the media components and additives disclosed herein. Any media containing any combination of components which is capable of initiating plb formation is included within the scope of the invention.

In one embodiment of this invention, the concentration of NAA is 2 mg/L and of BAP is 1 mg/L. In another embodiment, the concentration of NAA is 10 mg/L and of BAP is 1 mg/L. It is not intended that the present invention be limited by the concentration of NAA and/or BAP. The invention is also not limited to the use of NAA and/or BAP. Where NAA and BAP are used in the media, the concentrations of NAA and BAP may be varied such that the formation of differentiated structures (*e.g.*, shoots) is minimized, while the formation of plbs is maximized.

In one embodiment of the invention, plbs are initiated in liquid medium. It is preferred that plbs are initiated in liquid medium rather than on solidified (*e.g.*, agar-solidified) medium since plbs on agar-solidified medium typically turn brown or

differentiate into plants. It is also preferred, though not required, that liquid cultures be mixed gently by shaking on a gyrotory shaker in order to reduce the probability of re-orientation of cells of the largely undifferentiated totipotent body into a more differentiated structure.

Pineapple callus may be generated using methods known in the art such as those described by Mathews *et al.* (1981), *supra*. Briefly, pineapple callus is initiated from the basal region of in vitro-obtained shoot buds on MS medium supplemented with 4.5 mg/L NAA, 5.2 mg/L IAA (indoleacetic acid), 2.1 mg/L kinetin and maintained on MS medium with 400 mg/L casein hydrolysate, 15% (v) coconut water, 10 mg/L NAA. Such callus cultures, when grown on MS medium devoid of any growth regulators, regenerated shoot buds and optimum regeneration was obtained on MS medium containing 5% coconut water.

B. Maintenance In *in vitro* Culture Of Totipotent Bodies

The invention provides methods for the maintenance of pineapple totipotent bodies in *in vitro* culture. The terms "maintained" and "maintenance" refer to the retention, following generation as described *supra*, of viable and dividing cells of totipotent bodies. The methods of the invention are not limited to the maintenance of totipotent bodies which are generated in liquid culture. The methods of this invention also contemplate the maintenance of totipotent bodies (*e.g.*, plbs and callus) which are generated on solidified (*e.g.*, agar solidified) medium. The continued presence of viable and dividing cells in the totipotent body may be determined, for example, visually by observing an increase in size, measuring the volume of the totipotent body, observing incorporation of dyes such as tetrazolium chloride, *etc.*

Pineapple plbs and callus may be maintained in *in vitro* culture prior to and/or subsequent to transformation with a nucleic acid sequence of interest. Data provided herein demonstrates that pineapple plbs may be maintained in liquid medium for at least one year. In a preferred embodiment, plbs were maintained in liquid PI medium (*i.e.*, modified MS medium containing 2 mg/L NAA and 1 mg/L BAP).

The nature of the components of the medium used for the maintenance of plbs is not a critical aspect of this invention so long as viable and dividing cells of totipotent bodies are maintained in the medium and so long as the medium is a liquid medium. The term "liquid medium" as used herein refers to a medium which is in a state other than a solid state. Thus, a liquid medium may include a semi-solid medium (*e.g.*, solidified by addition of an amount equal to or less than 0.2% (w/v) phytagel, or an amount less than 0.8% agar).

Media components may be natural (*e.g.*, enzymatic digest, coconut milk, etc.) or artificial (*e.g.*, salts and growth regulators of defined chemical composition and amount). Generally, a suitable medium furnishes minerals, nitrogen sources, vitamins, growth factors, and the like. Components and methods for the preparation of plant culture media are commercially available, and are well within the ordinary skill in the art [see, *e.g.*, "Handbook of Cell Culture," Vol 2 and Vol. 3, W.R. Sharp, D.A. Evans, P.V. Ammirato, Y. Yamada (Eds.) (1983) MacMillan Publishing Co., New York].

It is preferred, though not necessary, that the liquid culture used for the maintenance of pineapple totipotent bodies be shaken gently, *e.g.*, on a gyrotory shaker; mixing of the liquid culture minimizes the re-orientation of cells of the undifferentiated totipotent body into a differentiated structure thus allowing the maintenance of undifferentiated structures over a longer period of time. In one preferred embodiment, plbs were maintained by subculturing to fresh PI liquid medium every 2 to 3 weeks and by selecting vigorous growing globular shape bodies. Cultures were maintained on a gyrotory shaker (80-90 rpm) at 27°C with a 16 h photoperiod with 35 $\mu mol.m^{-2}.sec^{-1}$ PAR provided by 40 watt white fluorescent and Sylvania Gro-Lux lamps (GTE Corp., Danvers, MA).

Importantly, plbs were successfully maintained in liquid PI medium as described *supra*, but were difficult to maintain on PI medium which had been solidified with agar since the latter resulted in regeneration of plants or in browning of the tissue culture.

C. Generation of Transgenic Pineapple Totipotent Bodies

A transgenic pineapple totipotent body may be generated by introducing any nucleic acid sequence of interest into a pineapple totipotent body. The transgenic pineapple totipotent bodies of the invention are not limited to totipotent bodies in which each and every cell expresses the nucleic acid sequence of interest. Rather, the invention includes within its scope any pineapple totipotent body which contains at least one cell which contains and is capable of expressing (or which expresses) the nucleic acid sequence of interest. Where it is desirable to express the nucleic acid sequence of interest in cells of an totipotent body, the nucleic acid sequence of interest is introduced into the cells of the totipotent body as part of an expression construct.

1. Expression Constructs

In one embodiment, the methods of the invention involve transformation of pineapple plbs with expression constructs in which the β-glucuronidase (GUS) gene is under the transcriptional control of a 35S promoter, double 35S promoter, rice actin-1 promoter, maize ubiquitin promoter, Emu promoter, scpepcd1 promoter, scpecd2 promoter, scrbcs1 promoter, scrbcs3 promoter, tarin gene promoter, or β-amylase promoter. In another embodiment, expression of the cowpea trypsin inhibitor (CpTi) gene is under the control of a cauliflower mosaic virus (CaMV) 35S RNA promoter sequence. In yet another embodiment, nucleic acid sequences encoding thaumatin protein are controlled by a double CaMV 35S promoter sequence. In a further embodiment, the 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase gene sequence is under the transcriptional control of a CaMV 35S promoter sequence.

The methods of the invention are not limited to the expression constructs disclosed herein. Any expression construct which is capable of introducing a nucleic acid sequence of interest into a pineapple plb cell is contemplated to be within the scope of this invention. Typically, expression constructs comprise the nucleic acid sequence of interest as well as companion sequences which allow the transcription of this sequence, and which allow cloning of the construct into a bacterial or phage host. The construct preferably, though not necessarily, contains an origin of replication

which is functional in a broad range of prokaryotic hosts. A selectable marker is generally, but not necessarily, included to allow selection of cells bearing the desired construct.

The invention is not limited to the promoter sequences used herein. Any promoter sequence which is capable of directing expression of an operably linked nucleic acid sequence of interest in a pineapple plb cell is contemplated to be within the scope of the invention. Promoters include, but are not limited to, promoter sequences of bacterial, viral and plant origins. Promoters of bacterial origin include, but are not limited to, the octopine synthase promoter, the nopaline synthase promoter and other promoters derived from native Ti plasmids. Viral promoters include, but are not limited to, the 35S and 19S RNA promoters of cauliflower mosaic virus (CaMV), and T-DNA promoters from *Agrobacterium*. Plant promoters include, but are not limited to, the ribulose-1,3-bisphosphate carboxylase small subunit promoter, maize ubiquitin promoters, the phaseolin promoter, the E8 promoter, and the Tob7 promoter.

The invention is not limited to the number of promoters used to control expression of a nucleic acid sequence of interest. Any number of promoters may be used so long as expression of the nucleic acid sequence of interest is controlled in a desired manner. The use of two or more promoters for the control of gene expression is known in art, and is exemplified herein by the double 35S RNA promoter of CaMV (Table 4; Figure 1). Chimeric promoters which are derived from different genes are also included within the scope of the invention. For example, a chimeric promoter containing a trimer of the octopine synthase (*ocs*) upstream activating sequence ligated to the mannopine synthase (*mas2'*) promoter/activator has been previously described (Ni *et al.* (1995) The Plant J. 7:661-676).

Promoters may control constitutive or inducible expression. Examples of environmental conditions that may effect transcription by inducible promoters include anaerobic conditions or the presence of light. Examples of promoters under developmental control include promoters that initiate transcription only in response to ethylene, such as E8 and Tob7. Furthermore, the selection of a promoter may be governed by the desirability that expression be over the whole plant, or localized to

selected tissues of the plant, *e.g.*, root, leaves, fruit, *etc.* For example, promoters active in flowers are known (Benfy *et al.* (1990) Plant Cell 2:849-856).

The promoter activity of any nucleic acid sequence in pineapple plb cells may be determined (*i.e.*, measured or assessed) using methods well known in the art and exemplified herein. For example, a candidate promoter sequence may be tested by ligating it in-frame to a reporter gene sequence to generate a reporter construct, introducing the reporter construct into pineapple plbs using methods described herein, and detecting the expression of the reporter gene (*e.g.*, detecting the presence of encoded mRNA or encoded protein, or the activity of a protein encoded by the reporter gene). The reporter gene may confer antibiotic or herbicide resistance. Examples of reporter genes include, but are not limited to, *dhfr* which confers resistance to methotrexate [Wigler M *et al.*, (1980) Proc Natl Acad Sci 77:3567-70]; *npt*, which confers resistance to the aminoglycosides neomycin and G-418 [Colbere-Garapin F *et al.*, (1981) J. Mol. Biol. 150:1-14] and *als* or *pat*, which confer resistance to chlorsulfuron and phosphinotricin acetyl transferase, respectively. Recently, the use of a reporter gene system which expresses visible markers has gained popularity with such markers as β-glucuronidase and its substrate (X-Gluc), luciferase and its substrate (luciferin), and β-galactosidase and its substrate (X-Gal) being widely used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system [Rhodes CA *et al.* (1995) Methods Mol Biol 55:121-131]. In a preferred embodiment, the reporter gene is a GUS gene.

In addition to a promoter sequence, the expression construct preferably contains a transcription termination sequence downstream of the nucleic acid sequence of interest to provide for efficient termination. In one embodiment, the termination sequence is the nopaline synthase (NOS) sequence. In another embodiment the termination region comprises different fragments of sugarcane ribulose-1,5-biphosphate carboxylase/oxygenase (rubisco) small subunit (scrbcs) gene. The termination sequences of the expression constructs are not critical to the invention. The

termination sequence may be obtained from the same gene as the promoter sequence or may be obtained form different genes.

If the mRNA encoded by the nucleic acid sequence of interest is to be efficiently translated, polyadenylation sequences are also commonly added to the expression construct. Examples of the polyadenylation sequences include, but are not limited to, the *Agrobacterium* octopine synthase signal, or the nopaline synthase signal. Where it is preferred that the nucleic acid sequence of interest is not translated into a polypeptide, *e.g.*, where the nucleic acid sequence of interest encodes an antisense RNA, polyadenylation signals are not necessary.

Constructs for the transformation of pineapple plbs are not limited to the type or nature of the expressed genes disclosed herein. Any nucleic acid sequence whose introduction into a pineapple plb cell may be deemed of interest to one of ordinary skill in the art may be used to create transgenic pineapple plbs. Nucleic acid sequences of interest include sequences which encode a polypeptide sequence whose expression may be deemed useful by one of skill in the art. For example, it may be desirable to express a nucleic acid sequence which encodes a polypeptide sequence having, for example, enzyme activity. One example of such an enzyme is the ACC deaminase enzyme which metabolizes ACC in plant tissue thereby lowering the level of ethylene which is responsible for fruit ripening (U.S. Patent No. 5,512,466, the contents of which are hereby incorporated by reference). Another enzyme which may be desirably expressed in pineapple is the sucrose phosphate synthase enzyme which increases the level of sucrose in the fruit. The nucleic acid sequence of the gene encoding this enzyme is known [*e.g.*, in maize; Worrell *et al.* (1991) Plant Cell 3:1121-1130] (Figure 14) (SEQ ID NO:2) and has been assigned GenBank accession number m97550. Yet another example of a protein whose expression in pineapple may be desirable is the sweetness protein. The nucleic acid sequence for the gene encoding the sweetness protein is known in the art (see, *e.g.*, U.S. Patent Application No. 08/670,186, the contents of which are herein incorporated by reference). Transformation of pineapple plants with the sweetness protein is useful in, for example, providing a base level of sweetness in the fruit, thus reducing the effects of

differences in fruit maturity by providing more uniform sweetness from the bottom to the top of the fruit.

Alternatively, it may be desirable to express a nucleic acid sequence which encodes an antisense RNA that hybridizes with a genomic plant DNA sequence. For example, it may be of advantage to express antisense RNA which is specific for genomic plant DNA sequences that encode an enzyme whose activity is sought to be decreased. Examples of DNA sequences whose reduced expression may be desirable are known in the art including, but not limited to, the ethylene inducible sequences in fruit (U.S. Patent No. 5,545,815, the entire contents of which are herein incorporated by reference). Expression of antisense RNA which is homologous with these ethylene inducible sequences is useful in delaying fruit ripening and in increasing fruit firmness. Other DNA sequences whose expression may be desirably reduced include the ACC synthase gene which encodes the ACC synthetase enzyme that is the first and rate limiting step in ethylene biosynthesis. Nucleic acid sequences for this gene have been described from a number of plant sources (*e.g.*, Picton *et al.* (1993) The Plant J. 3:469-481; U.S. Patent No. 5,365,015, the contents of which are herein incorporated by reference) and may be used to select a pineapple ACC synthase gene from a pineapple genomic library using methods well known in the art (Sambrook, J. *et al.*, (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York). Expression of antisense RNA which hybridizes with ACC synthase genomic sequences in pineapple plants may be desirable to delay fruit ripening. Yet another sequence whose expression may be advantageously reduced is the genomic sequence encoding the enzyme polyphenol oxidase. This enzyme is involved in the browning reaction that occurs during chilling injury. Nucleic acid sequences encoding this enzyme have been previously described in the art (*e.g.*, Shahar et al. (1992) Plant Cell 4:135-147], is shown in Figure 13 (SEQ ID NO:2), and has been assigned GenBank accession number s40548. The use of antisense polyphenol antisense sequences has been reported to inhibit polyphenol oxidase (PPO) gene expression and to inhibit browning [Bachem *et al.* (1994) Bio/Technology 12:1101-

1105]. One of skill in the art knows that the antisense DNA segment to be introduced into the plant may include the full length coding region of the targeted gene or a portion thereof. Complete homology between the nucleotide sequences of the antisense RNA and the targeted genomic DNA is not required. Rather, antisense DNA sequences which encode antisense RNA sequences that are partially homologous to a targeted genomic DNA sequence are contemplated to be within the scope of the invention so long as the antisense RNA sequences are capable of repressing expression of the target genomic DNA sequence.

The invention is not limited to constructs which express a single nucleic acid sequence of interest. Constructs which contain a plurality of (*i.e.*, two or more) nucleic acid sequences under the transcriptional control of the same promoter sequence are expressly contemplated to be within the scope of the invention. Such constructs may be desirable, for example, where the expression products of the plurality of nucleic acid sequences contained within the construct provide protection against different pathogens, and where simultaneous protection against these different pathogens is deemed advantageous. Also included within the scope of this invention are constructs which contain the same or different nucleic acid sequences under the transcriptional control of different promoters. Such constructs may be desirable to, for example, target expression of the same or different nucleic acid sequences of interest to selected plant tissues.

Expression constructs may be introduced into plbs using methods known in the art. In one embodiment, the expression constructs are introduced into pineapple plbs by particle mediated gene transfer. Particle mediated gene transfer methods are known in the art, are commercially available, and include, but are not limited to, the gas driven gene delivery instrument descried in McCabe, U.S. Patent No. 5,584,807, the entire contents of which are herein incorporated by reference. This method involves coating the nucleic acid sequence of interest onto heavy metal particles, and accelerating the coated particles under the pressure of compressed gas for delivery to the target tissue.

Other particle bombardment methods are also available for the introduction of heterologous nucleic acid sequences into plbs. Generally, these methods involve depositing the nucleic acid sequence of interest upon the surface of small, dense particles of a material such as gold, platinum, or tungsten. The coated particles are themselves then coated onto either a rigid surface, such as a metal plate, or onto a carrier sheet made of a fragile material such as mylar. The coated sheet is then accelerated toward the target biological tissue. The use of the flat sheet generates a uniform spread of accelerated particles which maximizes the number of cells receiving particles under uniform conditions, resulting in the introduction of the nucleic acid sample into the target tissue.

Alternatively, an expression construct may be inserted into the genome of pineapple plb cells by infecting plbs with a bacterium, including but not limited to an *Agrobacterium* strain previously transformed with the nucleic acid sequence of interest. Generally, disarmed *Agrobacterium* cells are transformed with recombinant Ti plasmids of *Agrobacterium tumefaciens* or Ri plasmids of *Agrobacterium rhizogenes* (such as those described in U.S. Patent No. 4,940,838, the entire contents of which are herein incorporated by reference) which are constructed to contain the nucleic acid sequence of interest using methods well known in the art (Sambrook, J. *et al.*, (1989) *supra*). The nucleic acid sequence of interest is then stably integrated into the plant genome by infection with the transformed *Agrobacterium* strain. For example, heterologous nucleic acid sequences have been introduced into plant tissues using the natural DNA transfer system of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* bacteria (for review, see Klee *et al.* (1987) Ann. Rev. Plant Phys. 38:467-486).

One of skill in the art knows that the efficiency of transformation by *Agrobacterium* may be enhanced by using a number of methods known in the art. For example, the inclusion of a natural wound response molecule such as acetosyringone (AS) to the *Agrobacterium* culture has been shown to enhance transformation efficiency with *Agrobacterium tumefaciens* [Shahla et al. (1987) Plant Molec. Biol.

8:291-298]. Alternatively, transformation efficiency may be enhanced by wounding the target tissue to be transformed. Wounding of plant tissue may be achieved, for example, by punching, maceration, bombardment with microprojectiles, etc. [see, *e.g.*, Bidney *et al.* (1992) Plant Molec. Biol. 18:301-313].

It may be desirable to target the nucleic acid sequence of interest to a particular locus on the plant genome. Site-directed integration of the nucleic acid sequence of interest into the plant cell genome may be achieved by, for example, homologous recombination using *Agrobacterium*-derived sequences. Generally, plant cells are incubated with a strain of *Agrobacterium* which contains a targeting vector in which sequences that are homologous to a DNA sequence inside the target locus are flanked by *Agrobacterium* transfer-DNA (T-DNA) sequences, as previously described (Offringa *et al.*, (1996), U.S. Patent No. 5,501,967, the entire contents of which are herein incorporated by reference). One of skill in the art knows that homologous recombination may be achieved using targeting vectors which contain sequences that are homologous to any part of the targeted plant gene, whether belonging to the regulatory elements of the gene, or the coding regions of the gene. Homologous recombination may be achieved at any region of a plant gene so long as the nucleic acid sequence of regions flanking the site to be targeted is known.

Where homologous recombination is desired, the targeting vector used may be of the replacement- or insertion-type (Offringa *et al.* (1996), *supra*). Replacement-type vectors generally contain two regions which are homologous with the targeted genomic sequence and which flank a heterologous nucleic acid sequence, *e.g.*, a selectable marker gene sequence. Replacement type vectors result in the insertion of the selectable marker gene which thereby disrupts the targeted gene. Insertion-type vectors contain a single region of homology with the targeted gene and result in the insertion of the entire targeting vector into the targeted gene.

Other methods are also available for the introduction of expression constructs into plant tissue, *e.g.*, electroinjection (Nan *et al.* (1995) In "Biotechnology in Agriculture and Forestry," Ed. Y.P.S. Bajaj, Springer-Verlag Berlin Heidelberg, Vol

34:145-155; Griesbach (1992) HortScience 27:620); fusion with liposomes, lysosomes, cells, minicells or other fusible lipid-surfaced bodies (Fraley *et al.* (1982) Proc. Natl. Acad. Sci. USA 79:1859-1863; polyethylene glycol (Krens *et al.* (1982) nature 296:72-74); chemicals that increase free DNA uptake; transformation using virus, and the like.

2. Selection of Transgenic Pineapple Totipotent Bodies

Pineapple totipotent bodies transformed with a heterologous nucleic acid sequence of interest are readily detected using methods known in the art including, but not limited to, restriction mapping of the genomic DNA, PCR-analysis, DNA-DNA hybridization, DNA-RNA hybridization, DNA sequence analysis and the like.

Additionally, selection of transformed totipotent bodies may be accomplished using a selection marker gene. It is preferred, though not necessary, that a selection marker gene be used to select transformed plbs. A selection marker gene may confer positive or negative selection.

A positive selection marker gene may be used in constructs for random integration and site-directed integration. Positive selection marker genes include antibiotic resistance genes, and herbicide resistance genes and the like. In one embodiment, the positive selection marker gene is the NPTII gene which confers resistance to geneticin (G418) or kanamycin. In another embodiment the positive selection marker gene is the HPT gene which confers resistance to hygromycin. The choice of the positive selection marker gene is not critical to the invention as long as it encodes a functional polypeptide product. Positive selection genes known in the art include, but are not limited to, the ALS gene (chlorsulphuron resistance), and the DHFR-gene (methothrexate resistance).

A negative selection marker gene may also be included in the constructs. The use of one or more negative selection marker genes in combination with a positive selection marker gene is preferred in constructs used for homologous recombination. Negative selection marker genes are generally placed outside the regions involved in the homologous recombination event. The negative selection marker gene serves to provide a disadvantage (preferably lethality) to cells that have integrated these genes

into their genome in an expressible manner. Cells in which the targeting vectors for homologous recombination are randomly integrated in the genome will be harmed or killed due the presence of the negative selection marker gene. Where a positive selection marker gene is included in the construct, only those cells having the positive selection marker gene integrated in their genome will survive.

The choice of the negative selection marker gene is not critical to the invention as long as it encodes a functional polypeptide in the transformed pineapple plbs. The negative selection gene may for instance be chosen from the aux-2 gene from the Ti-plasmid of *Agrobacterium*, the *tk*-gene from SV40, cytochrome P450 from *Streptomyces griseolus*, the *Adh*-gene from Maize or Arabidopsis, *etc.* Any gene encoding an enzyme capable of converting a substance which is otherwise harmless to pineapple plb cells into a substance which is harmful to pineapple plb cells may be used.

C. Generation of Transgenic Pineapple Plants

The present invention provide transgenic pineapple plants. The transgenic pineapple plants of the invention are not limited to plants in which each and every cell expresses the nucleic acid sequence of interest. Included within the scope of this invention is any pineapple plant which contains at least one cell which expresses the nucleic acid sequence of interest. It is preferred, though not necessary, that the transgenic plant express the nucleic acid sequence of interest in more than one cell, and more preferably in one or more tissue.

Transgenic pineapple plants may be generated from transformed totipotent bodies using, for example, methods described herein. For example, where transgenic plbs are used to regenerate transgenic plants, shoots are first generated by transferring plbs onto the surface of agar solidified (*e.g.*, 0.8% Bacto agar) hormone-free medium or PM2 (*i.e.*, 2 mg/L BAP) medium. Roots are then generated by transferring the structures containing shoots onto the surface of agar-solidified (*e.g.*, 0.8% Bacto agar) medium containing 1 mg/L NAA. The resulting structures are potted in soil in pots

and placed in a greenhouse. Once the plants reach a size suitable for transfer, they may be transferred to the field for further growth and development

Alternatively, where transgenic callus is used to regenerate transgenic plants, plants are regenerated by the method described by Mathews et al. (1981) as described supra.

Transformation of pineapple plants with the nucleic acid sequence of interest may be determined using any number of methods known in the art, *e.g.*, restriction mapping of genomic DNA, PCR analysis, DNA-DNA hybridization, DNA-RNA hybridization, DNA sequence analysis, etc. Additionally, transformation may be determined by visual inspection and comparison of transgenic and wild-type plants. the phenotype inspected depends on the phenotypic characteristics expected to be conferred by the nucleic acid sequence of interest used in the transformation of the plants. Examples of phenotypic characteristics may include fruit flesh color, fruit tissue firmness, leaf color, fruit yield, plant vigor, *etc.*

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: g (gram); mg (milligrams); µg (microgram); M (molar); mM (milliMolar); µM (microMolar); nm (nanometers); L (liter); ml (milliliter); µl (microliters);°C (degrees Centigrade); m (meter); sec. (second); DNA (deoxyribonucleic acid); cDNA (complementary DNA); RNA (ribonucleic acid); mRNA (messenger ribonucleic acid); X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside); LB (Luria Broth), PAGE (polyacrylamide gel electrophoresis); NAA (α-naphtaleneacetic acid); BAP (6-benzyl aminopurine); Tris (tris(hydroxymethyl)-aminomethane); PBS (phosphate buffered saline); 2 X SSC (0.3 M NaCl, 0.03 M Na_3citrate, pH 7.0); Agri-Bio Inc. (North Miami, FL); Analytical Scientific Instruments (Alameda, CA); BioRad (Richmond, CA); Clontech (Palo Alto CA); Delmonte Fresh Produce (Kunia, Hawaii); Difco

Laboratories (Detroit, MI); Dole Fresh Fruit (Wahiawa, Hawaii); Dynatech Laboratory Inc. (Chantilly VA); Gibco BRL (Gaithersburg, MD); Gold Bio Technology, Inc. (St. Louis, MO); GTE Corp. (Danvers, MA); MSI Corp. (Micron Separations, Inc., Westboro, MA); Pioneer Hi-Bred International, Inc. (Johnston, IA); 5 Prime 3 Prime (Boulder, CO); Sigma (St. Louis, MO); Promega (Promega Corp., Madison, WI); Stratagene (Stratagene Cloning Systems, La Jolla, CA); USB (U.S. Biochemical, Cleveland, OH).

EXAMPLE 1

Generation of Pineapple Protocorm-like Bodies (plbs) And Plants Derived Therefrom

Pineapple plbs were initiated from crown and vegetative shoot explants, maintained for several months and used to generate pineapple plants which showed low phenotypic variation as compared to pineapples generated by traditional shoot regeneration methods from callus.

A. Initiation of protocorm-like bodies (plbs)

The suitability of plbs and *in vitro* shoot cultures for the production of pineapple plants with a minimum of spontaneous mutations was compared. Two cultivars, F153 (Delmonte Fresh Produce) and D10 (Dole Fresh Fruit) of field-grown pineapple plants (*Ananas cosmosus* (L.) Merr. variety Smooth Cayenne) were used to establish the explant-derived *in vitro* tissue cultures.

For plb generation, crowns from the F153 cultivar were selected as explants since plants generated from callus produced from crowns have been previously reported to exhibit lesser somatic variation than plants regenerated from callus derived from other types of explants (Wakasa (1979) Japan J. Breed. 29:13-22). Also for plb generation, vegetative shoots from the D10 cultivar were used to determine whether the phenotypic variation of plb-derived plants was affected by the tissue source from which plbs were initiated. Vegetative shoot *in vitro* cultures were initiated to generate

plants from D10 since this method is the traditionally used *in vitro* method for regeneration of pineapple plants.

Explants were obtained by removing all the leaves except the inner 3 to 4 leaves from crowns of 16- to 17-month-old crop plants, or from 3- to 6-month-old vegetative shoots. The suitability of different "CLOROX" (5.25% sodium hypochlorite solution) concentrations and durations of treatment for surface sterilization of crowns and vegetative shoots from F153 was investigated. Generally, sterilization with 20 or 50% "CLOROX" for 30 min gave 22.9% success rate on sectioned explants from F153 crowns and 14.7% success rate on sectioned explants from F153 vegetative shoots.

Plbs, whether derived from crowns or shoots, were initiated in liquid culture without the use of agar solidified medium. Without limiting the invention to any particular theory, liquid culture and shaking is advantageous for the initiation and maintenance of pineapple plbs since these conditions are believed to reduce the orientation of cells. Plbs are a group of polarized cells predetermined as apical meristems which will become shoots. Shaking in liquid culture permits initiation and maintenance of plb cells which are not fully differentiated as leaf primodia.

Various combinations of NAA and BAP concentrations were tested to increase plb induction as shown in Table 1.

TABLE 1

Short-term responses of young pineapple shoots to NAA and BAP combinations in liquid medium		
NAA / BAP (mg/l)	Responses	% Increase in Size
0 / 0	light-pigmented shootings	17
2 / 1	light-pigmented shootings with few plbs	77

5 / 1	light-pigmented shootings with some plbs	70
10 / 1	mostly plbs	23
20 / 1	mostly plbs with some necrosis	30

Plb cultures from F153 crowns were initiated by placing surface sterilized crown explants in liquid PI medium [liquid modified MS medium: MS salts (Gibco BRL), 100 mg/L myo-inositol (Sigma), 1 mg/L thiamine-HCl (Sigma), 30 g sucrose (Sigma), pH 5.6-5.8: supplemented with 2 mg/L α-naphtaleneacetic acid (NAA) (Sigma) and 1 mg/L 6-benzyl aminopurine (BAP) (Sigma)] or in liquid modified MS media with 10 mg/L NAA and 1 mg/L BAP. The culture was maintained on a gyratory shaker at 80-90 rpm at 27°C with a 16 h photoperiod with 35 $\mu mol.m^{-2}.sec^{-1}$ Photosynthetically Active Radiation (PAR) (μEinstein or μE) provided by 40 watt white fluorescent and Sylvania Gro-Lux lamps (GTE Corp.). New growth of shoots and globular cell structure was observed 2 to 4 weeks after culture initiation. Uniform protocorm-like bodies (plbs) were obtained by transferring the globular structures to fresh PI medium (*i.e.*, modified MS medium containing 2 mg/L NAA and 1 mg/L BAP) every 2-3 weeks.

Plb cultures from D10 vegetative shoots were initiated through shoot multiplication in PM2 (2 mg/L BAP in liquid modified MS medium). This culture resulted in plbs which were more organized than the plbs derived from F153 crowns, and which also contained shoots. Transfer of these plbs (herein referred to as "shoot plbs") to PI medium (*i.e.*, modified MS medium containing 2 mg/L NAA and 1 mg/L BAP) resulted within a few weeks in dedifferentiated plbs which were similar to the plbs obtained from F153 crowns. These plbs were maintained (*i.e.*, shoot plbs were not produced) in PI by transferring to fresh medium every 3 weeks.

Shoot cultures were initiated from D10 by placing explants in liquid PM2 medium. Transfer to a differentiation medium was not required since the explants

produced differentiated shoots. Shoot cultures were maintained by cutting new lateral shoots and transferring them to fresh liquid PM2 medium every 3-4 weeks.

B. Maintenance of plbs

All tissue handling and culture was conducted under sterile conditions. The conditions for the maintenance of plbs are different from those of callus cultures (Mathews and Rangan (1981) Scientia Hort. 11:319-328). Plbs generated from crowns or vegetative shoots as described *supra* were maintained in liquid PI medium on a gyrotory shaker. Plbs were difficult to maintain on agar-solidified PI medium containing 0.8% agar (Bacto agar; Difco Laboratories) since the tissue turned brown or generated plants. Plbs were successfully maintained for at least one year by sub-culturing under the same conditions as plb initiation to fresh PI liquid medium every 2 to 3 weeks and by selecting (visually and by measuring the volume of plbs) vigorous growing light creamy yellow globular shaped bodies.

C. Regeneration of plants from plbs

Plants were regenerated from plbs in two steps. The first step involved initiation of shoots, while the second step involved generation of roots. Shoots were generated by transferring plbs onto hormone-free agar-modified MS medium or onto agar-PM2. Each clump (approximately 15 mm^3 in size, *i.e.*, 3-5 mm diameter) produced an average of five shoots 2- to 10-mm long in 2 months. Roots were then initiated by transferring the shoots after 3-4 weeks to liquid modified MS medium containing 1 mg/L NAA. This resulted in the formation of approximately 6-8 roots of approximately 2-3 cm length per plant. Root initiation in liquid culture was preferred since roots developed on agar-modified MS medium were often injured or broken when the plant was transferred from agar-solidified medium to soil. These conditions resulted in rooting of approximately 97-99% of the shoots and the generation of plants (*i.e.*, structure containing shoots and roots). Approximately 150 plants from each of the two pineapple cultivars, F153 and D10, were regenerated from plbs.

D. Evaluation of plants derived from plbs

To compare phenotypic variation between plants generated from plbs and from *in vitro* shoot multiplication, plants which were regenerated from plbs of each of the F153 (150 plants) and D10 (150 plants) pineapple cultivars and from F153 (154 plants) and D10 (30,000 plants) *in vitro* shoot multiplication were planted in pots containing soil in a greenhouse and grown for about 3 months until the resulting plants reached 3 inches in height. Leaves of the potted plants were morphologically evaluated for leaf variegation, spininess, and malformations.

For plants generated from F153 crown-derived plbs, 75.4% of the plants had normal morphology, 22.5% had leaf spine, and 2.1% were dwarf. For plants generated from D10 vegetative shoot-derived plbs, 99% of the plants were normal, while 1% were variegated. For plants derived from F153 *in vitro* shoot multiplication, 96.1% were normal and 3.9% showed spininess in the leaves. For plants derived from D10 *in vitro* shoot multiplication, over 99.9% were normal and the remaining 0.1% showed variegated leaves. These results demonstrate that phenotypic variation (*i.e,* 1% in D10, and 24.6% in F153) of plants derived from plbs was greater than the variation (*i.e.*, 3.9% for F153 and 0.1% for D10) in plants derived from *in vitro* shoot multiplication.

In order to also compare the reproducibility of the above-observed morphological variation, the morphology of plants was compared between F153 pineapple plants which were derived from shoots or from plbs which had been cultured for approximately one year. Plant morphology was assessed after growth for 5-7 months after transplantation in a greenhouse. The results are shown in Table 2.

TABLE 2

Morphology Of F153 Pineapple Plants Derived From Shoots and From Plbs

	Shoot multiplied No. of plants (%)				Plb-derived No. of plants (%)			
Month after planting	0	6	12		0	4.5	10.5	
Normal	132	148	149	(97)	99	107	99	(70)
Spiny leaves	17	6	5	(3)	34	32	38	(27)
Multiple shoots	4	0	0	(0)	4	0	0	(0)
Dwarf	0	0	0	(0)	5	3	5	(3)
Total	154	154	154	(100)	142	142	142	(100)

The results in Table 2 demonstrate that while the proportion of pineapple plants which exhibit normal morphology and which are derived from cultured plbs is greater than the proportion derived from shoots, this proportion is nevertheless reproducible and sufficiently high to make pineapple plants derived from cultured plbs a commercially acceptable product.

In order to compare morphological traits of pineapple plants derived from plbs, crowns and *in vitro* multiplication of shoots, plant height, leaf weight length and width were compared in plants grown for one year in the field as shown in Table 3.

TABLE 3

Morphology of Pineapple Plants Derived From *in vitro* Shoots, Crowns and Protocorm-Like Bodies

	Shoot-multiplied vs. crown		protocorm-like body vs. crown	
Morphological trait	*In vitro* shoot	Crown	Plb	Crown
Plant height (cm)	93.8 ± 1.2	86.3 ± 3.2	89.5 ± 2.6	92.5 ± 1.0
D-leaf weight (g)	38.3 ± 1.6	42.0 ± 1.7	34.9 ± 1.6	39.8 ± 1.9
D-leaf length (cm)	76.8 ± 1.2	75.4 ± 1.7	72.7 ± 1.8	74.0 ± 2.1
D-leaf width (cm)	4.62 ± 0.10	4.98 ± 0.11	4.70 ± 0.10	*5.2 ± 0.05

* $P \leq 0.05$

Data presented in Table 3 shows that there was no significant difference in morphology between plants which were derived from *in vitro* shoots, crowns and protocorm-like bodies which had been cultured for one year. Additionally, fruit yield and quality (*e.g.*, acidity, sweetness, *etc.*) were also unaltered in pineapple fruit derived from *in vitro* shoots, from crowns and from protocorm-like bodies which had been cultured for one year (data not shown). These data also suggested that pineapple plants and fruit derived from cultured plbs are commercially useful.

Although plants derived from pineapple plbs of two cultivars uniformly showed greater phenotypic variation as compared to plants derived from *in vitro* shoot multiplication, and as compared to the previously reported phenotypic variation (from 7% to 100% depending on the starting explant tissue) of pineapple plants derived from callus (Wakasa (1979) *supra*) the suitability of pineapple plbs as targets for transformation was investigated.

EXAMPLE 2

Transformation of Pineapple Plbs With Fourteen Reporter Constructs Containing β-glucuronidase Reporter Gene

In order to determine the suitability of pineapple plbs as targets for genetic transformation, plbs derived from the F153 and D10 cultivars were transformed with plasmid constructs containing the β-glucuronidase (GUS) reporter gene and optionally contained either the neomycin phosphotransferase (NPTII) selection gene, or the hygromycin phosphotransferase (HPT) selection gene, under the transcriptional control of different promoters. Transformation of plb tissue was accomplished by using either a biolistic transformation method (also known as gene gun bombardment method), or by a *Agrobacterium*-mediated transformation method. Stable transformation was determined by selection in liquid PI medium of plants regenerated from transfected plbs on geneticin (G418) for expression of NPTII, or on hygromycin for expression of HPT.

A. Expression constructs

Fourteen expression constructs (Table 4) which contained heterologous gene under the transcriptional control of promoters from monocotyledous and dicotyledous plants were used to investigate the transformation potential of pineapple plbs. Figures 1-10, respectively, show the structures of plasmids pBI426, pBI121, pAct1-F, pAHC27, pHA9, pEmuGN, pWD1, pWD3, pML72, and pML75. The promoters in plasmids pBI426, and pBI121 are generally used for expression in dicotyledous plants, while the promoters in plasmids, pAct1-F, pAHC27, pHA9, pEmuGN, pWD1, pWD3, pML72, and pML75 are generally used for expression in monocotyledous plants. Plbs were transformed simultaneously with pHA9 and pAHC27 in order to simultaneously introduce into the plbs the NPTII selection marker gene of pHA9 and the GUS reporter gene of pAHC27.

TABLE 4
Biolistic Gun Transformation Of Pineapple Protocorm-like Bodies

Name	Promoter-Gene-Terminator	Helium Pressure (psi)	# Shots Mg DNA per shot	Transformation [a]	Sources and references
pBI426	35S:35S-GUS::NPTII-NOS	650-1800	1 or 2	Transient and stable	Ye *et al.* (1990) Plant Mol. Biol. 15:809-819; Datla *et al.* (1991) Gene 101:239-246; Kay *et al.* (1987) Science 236:1299-1300; National Research Council, Canada; Figure 1 herein.
pBI121	35S-GUS-NOS and NOS-NPTII-NOS	1100	1 or 2	Transient and stable	Clontech Catalogue No. 6018-2; Bevan *et al.* (1991) Nucleic Acids Research 12:8711-8721; Figure 2 herein.
pAct1-F	rice actin1-GUS-NOS	1100	1	Transient	McElroy *et al.* (1991) Mol. Gen. Genet. 231:150-160; Figure 3 herein.
pAHC27	maize ubiquitin-GUS-NOS	1100	1 or 2	Transient and stable	Christensen *et al.* (1992) Plant Mol. Biol. 18:675-689; U.S. Patent No. 5,510,474 the contents of which are incorporated by reference; Figure 4 herein.
pHA9	maize ubiquitin-NPTII-NOS	1100	1 or 2	Transient and stable	Christensen *et al.* (1992), *supra.*; Figure 5 herein.
pEmuGN	pEmu-GUS-NOS	1100	1	Transient	Last *et al.* (1991);Theor. Appl. Genet. 81:581-588; Figure 6 herein.
CD1GUS	scpepcd1-GUS-NOS	1100	1	Transient	Tang (1994) Hawaiian Sugar Planter's Association Annual Report pp. 10.
CD2GUS	scpepcd2-GUS-NOS	1100	1	Transient	Tang (1994) *supra.*.
pWD1	scrbcs1-GUS-NOS	1100	1	Transient	Tang *et al.*. (1996) In: Wilson, J.R. *et al.* (eds) Sugarcane: Research Towards Efficient and Sustainable Production, CSIRO, Brisbane, pp. 117-119; Figure 7 herein.
pWD3	scrbcs3-GUS-NOS	1100	1	Transient	Tang *et al.*. (1996) *supra.*; Figure 8 herein.

TABLE 4
Biolistic Gun Transformation Of Pineapple Protocorm-like Bodies

Name	Promoter-Gene-Terminator	Helium Pressure (psi)	# Shots Mg DNA per shot	Transformation [a]	Sources and references
R1	scrbcs1-GUS-scrbcs1 (550 bp)	1100	1	Transient	W. Tang, Ph. D. dissertation (UH, Dec. 1994)
R2	scrbcs1-GUS-scrbcs1 (500 bp)	1100	1	Transient	W. Tang, Ph. D. dissertation (UH, Dec. 1994)
R3	scrbcs1-GUS-scrbcs1 (370 bp)	1100	1	Transient	W. Tang, Ph. D. dissertation (UH, Dec. 1994)
R4	scrbcs1-GUS-scrbcs1 (200 bp)	1100	1	Transient	W. Tang, Ph. D. dissertation (UH, Dec. 1994)
pML72	tarin-GUS-NOS	1100	1 or 2	Transient	Figure 9 herein.
pML75	β-amylase-GUS-NOS	1100	1	Transient	Figure 10 herein.

[a] Transient transformation was determined by GUS staining of plbs 48 h after transformation. Stable transformation was determined by ELISA analysis of plant tissue for NPTII gene product.

Figure 3 shows the nucleotide sequence of the 5' transcribed region of the rice *Act*1-*Gus* gene fusion in p*Act1*-F between the *Act1* TATA box and *Gus* translation initiation codon. Nucleotides are numbered with the A of the *Act1* transcription initiation site designated as +1. Restriction sites are indicated and underlined. The different regions of the *Act1* 5'-transcribed sequences are: Upper case italic letters, *Act1* 5'-flanking region; Upper case letters, *Act1* exon sequence; lower case letters, *Act1* 5'-intron sequence; slashed lines, exon-intron splice sites, bold letters, *Act1* (5'-most) and *Gus* (3'-most) translation initiation codons. The translation product of the *Act1-Gus* junction region is shown below their respective codons.

B. Transformation of plbs

Pineapple plb tissue was used as target tissue for transformation using two approaches, *i.e.*, a gene gun bombardment method, and an *Agrobacterium*-mediated transformation method as described below.

1. Biolistic transformation and transient expression of GUS

Pineapple plbs were bombarded using the helium gas-driven microprojectile accelerator (PDS-1000/He) (Bio-Rad). Two to three days before bombardment, 20-30 plbs of 3-5 mm in diameter were placed in the 2.5-cm center of a 10-cm diameter petri plate containing modified MS medium supplemented with 0.8% Difco Bacto agar and 3% sucrose. Gold microcarriers (1.6 μm diameter) (Bio-Rad) were prepared by washing 3 times in absolute ethanol by vortexing for 1-2 min. and centrifuged at 14,000 x g for 10-20 sec. The resulting gold pellet was resuspended in 1 ml sterile dH_2O and stored at 4°C as 50 μl aliquots. Pre-washed gold microcarriers were coated with DNA constructs using the $CaCl_2$ precipitation method following the manufacturer's (Bio-Rad) instructions as modified by Sun *et al.* (1993) Sugar Cane 5;1-7. Each 50 μl gold suspension was mixed with 10-30 μg DNA, and 0.96 M $CaCl_2$, and 1.5 mM spermidine (Sigma) and vortexed for 3 min. After centrifugation in a microcentrifuge at 14,000 rpm for 10 sec., the liquid was removed and 250 μl

absolute ethanol added to resuspend the coated gold particles. After another centrifugation and aspiration, the coated gold microcarriers were resuspended in 60 µl absolute ethanol and 10 µl aliquots of the gold suspension used for each bombardment.

Several parameters, including helium pressure, number of shots per plate, and amount of DNA used per shot, were varied using plb as target material to determine the combination of conditions giving maximum levels of transient expression of the GUS reporter gene. Expression of GUS was detected by histochemical staining of tissue for GUS activity as previously described (Jefferson *et al.* (1987) EMBO J. 6:3901-3907) with the following modifications. Two days after bombardment, plant tissues were submerged in 500 mg-l^{-1} X-gluc (5-bromo-4-chloro-3-indolyl glucuronide) (Gold Bio Technology, Inc.) in GUS staining buffer (100 mM Na_2H/NaH_2PO_4, pH 7.0, 1% Triton-X, 1% DMSO, 10 mM EDTA) overnight at 37°C. In GUS-expressing cells, the *gus* gene product, β-glucuronidase, can cleave the colorless X-gluc to produce a blue precipitate. When necessary, chlorophyll was cleared by destaining the tissue with several changes of 70% ethanol. Transient expression was generally scored as the number of foci showing blue staining.

Transient expression was observed in plbs with each of the 14 constructs (Table 4). Among all the DNA constructs tested, pBI426 gave the highest transient GUS expression in plbs.

To determine the suitability and expression of different gene constructs using other pineapple tissues as targets from biolistic transformation, leaf, roots, fruit, flower petal and *in vitro* cultured shoots whose meristem areas were exposed by longitudinal cut halves or horizontal cut into disks were used as targets for biolistic transformation with the plasmids described in Table 4. *In vitro* shoots were unsuitable as targets for biolistic transformation since the number of GUS foci was very low. Transient expression of GUS following bombardment with pBI426 was observed when leaves and flower petals were used as target (Figure 11) but almost undetectable in target fruit and root tissue. In the fruit tissue, the strongest transient expression was observed following transformation with pML72 which contained the tarin promoter, a corm-specific promoter isolated from taro plants.

Two factors, including helium driving pressures (650 to 1800 psi), and amount of DNA used per shot, did not significantly affect the number of foci which transiently expressed the GUS gene, while duplicated shots almost doubled the number of transient GUS foci.

Based on the data from the bombardment of plbs, the optimized conditions for transient GUS gene expression were (a) using 10 μg of each DNA per bombardment; *i.e.* where more than one construct was used (*e.g.*, pHA9 and pAHC27), 10 μg of each construct was employed per bombardment; (b) positioning plb samples 7.5 cm below the stopping screen in the Biolistics device; (c) using helium gas at a pressure of 1100 psi or 1550 psi; and (d) using two shots per plate of plbs.

2. *Agrobacterium*-mediated transformation

For *Agrobacterium* transformation, plbs of 3-5 mm diameter were inoculated separately with two strains (LBA4301 and LBA4404) of *Agrobacterium tumefaciens*.

TABLE 5

List of *Agrobacterium* Strains Used for Pineapple plb Transformation

Name	Helper plasmid	T-DNA plasmid	Selection marker	Reporter marker
LBA4404	pAL4404	pBI121	NOS-NPTII	35S-GUS
LBA4301	pUCD2614	pUCD2716	35S-HPT	35S-GUS

Strain LBA4404 (Life Technologies Inc., GIBCO/BRL), is a disarmed octopine-type *Agrobacterium*, which has been previously described by Ooms *et al.* (1982) Plasmid 7:15-29, contains the disarmed Ti plasmid pBI121 which contains the selection marker NPTII under the control of the NOS promoter, as well as the GUS

reporter gene under the control of the CaMV double 35S promoter. Strain LBA4301 is a nopaline-type *Agrobacterium* strain which has been previously described by Klapwijk *et al.* (1979) Mol. Gen. Genet. 173:171-175 and Rogowsky *et al.* (1990) Plasmid 23:85-106] is derived from the C58 strain contains plasmid pUCD2716 which contains the HPT selection marker gene as well as the GUS reporter gene under the transcriptional control of the CaMV 35S promoter.

The inoculation process included microwounding by PDS-1000/He at 1100 psi, with 1.6 μm gold (Bio-Rad) and addition of *Agrobacterium* virulence inducer (100 μM acetosyringone) (Sigma) to the overnight grown bacterial culture two to three hours prior to the actual inoculation. Overnight bacterial suspension was 2X diluted with bacterial culture medium (LB) before inoculation. Plbs were immersed in diluted bacterial suspension for 5~10 min and were blotted dry on sterilized paper towels before co-cultivation. Co-cultivation was performed on 0.8% agar PM2 medium or in liquid PI medium for 2-3 days before transfer to media containing 200 mg/L cefotaxime (Agri-Bio Inc.) to kill *Agrobacterium*.

C. Selection of transformed plbs

One to two weeks after the plbs were bombarded or co-incubated with *Agrobacterium*, the plbs were transferred to liquid PI medium or 0.8% agar PM2 medium containing selection antibiotics. The antibiotic G418 (Sigma) was included to select for cells transformed with constructs containing the NPTII gene, while the antibiotic hygromycin (Sigma) was used to select cells transformed with constructs containing the HPT gene.

Figure 12 shows a representative antibiotic growth inhibition curve for F153 (Figure 12A and 12C) and for D10 (Figure 12B and 12D) plbs treated for one month (open circles) or two months (filled squares) with different G418 concentrations (mg/ml) in liquid PI media (Figures 12A and 12B) and agar-solidified shoot regeneration PM2 media (Figures 12C and 12D). The concentrations of antibiotics used were determined such that they resulted in killing (as judged by their necrotic

appearance) of more than 99% of plbs which were not transformed with the antibiotic resistance gene. For F153 plbs, 20-30 mg/L G418 and 20-30 mg/L hygromycin were used, respectively. For D10 plbs, 40-50 mg/L G418 and ~75 mg/L hygromycin were used, respectively.

Data presented herein shows that selection of plbs was more efficient in liquid media than on agar solidified media. Using G418, two to three months were required to kill non-transformed plbs on agar media in contrast to only one month in liquid media. This allowed for the more rapid selection of plbs which are transformed from those which are not (Figure 12).

Without limiting the invention to any particular theory, the observation that selection in liquid media is better than selection on agar-solidified media can be explained by the following. First, tissues are totally submerged and are in full contact with the antibiotic in liquid medium. Second, selection at the plb multiplication stage in liquid PI medium allows single transformed cells to form non-chimeric transformed plbs, since all cells appeared to be able to form new plbs, while selection of plb regenerating into shoots/plants on agar PM2 medium may lead to chimeric plants. This is highly probable since plbs are partially organized with a predetermined apex where the shoot emerges, and since the chances of the transformed cells locating at the meristematic region and in the correct layer of cells are very slim. Third, development of plbs in liquid is likely to be more synchronous than the development of plbs on agar. Synchronously developed plbs allow uniform stages of plants to be exposed to the selection chemicals in the medium thus resulting in a lower frequency of escapes of non-transformed lines.

D. Production of stably transformed transgenic pineapple plants from transformed plbs

Transgenic pineapple plb cultures which were selected in liquid media as described above following either biolistic bombardment alone or in combination with *Agrobacterium* transformation were transferred to 0.8% agar PM2 medium containing selection antibiotics to allow differentiation into plants. Transformed plbs selected in

liquid culture regenerated numerous (approximately 20-30) plants compared to the few individual plants (generally fewer than 5) regenerated during selection of transformed plbs on agar media immediately after bombardment.

For the biolistic bombardment method of transformation, stable expression was detected in plants generated from pineapple plbs which had been selected with antibiotic and which showed trangene GUS expression by detecting the presence of the *neo* gene product (*i.e.*, neomycin phosphtoransgerse II) using ELISA following the manufacturer's (5 Prime 3 Prime) protocol. Briefly, crude extracts were obtained by grinding a ratio of 1 g samples in 5-10X ml grinding buffer (0.25 M Tris-HCL, pH 7.8, 0.2% DIECA, 25 mg/L chymostatin, 50 mg/L leupeptin). Equal amounts of protein (20-50 μg per well) from the extracts were assayed. Absorbance of each well was measured against the reagent blank at a single wavelength (405 nm) using a MRX Microplate Reader (Dynatech Laboratory Inc.). Protein was quantified by a modified Bradfrod dye-binding procedure following the manufacturer's (BioRad) instructions.

Plbs selected in liquid media during biolistic transformation separately with each of pBI426 and pBI121, and co-transformed with pHA9 and pAHC27 gave rise to stably transformed plbs as determined by their antibiotic resistance and ELISA. For example, after 3 months of liquid media selection, randomly selected plbs bombarded separately with pBI426 and pAHC27 showed 2 to 5 times greater expression of NPTII protein than the unbombarded controls as measured by NPTII ELISA.

Similarly, plants regenerated from pBI426- and pBI121-transformed plbs expressed high levels of the antibiotic resistance gene products as determined by ELISA. Expressed levels of NPTII in plb-derived plants transformed with the double 35S promoter-driver fused GUS::NPTII gene in pBI426 were double the levels of expressed NPTII in plb-derived plants transformed with the NOS promoter-driven NPTII gene in pBI121. Stable transformation was also observed in plants generated from plbs transformed separately with pAHC27 and pHA9.

Southern hybridization was conducted in order to determine whether the expression of NPTII in plants regenerated from plbs which had been transformed by particle bombardment with pBI426 and pBI121 was the result of integration of the

transgene encoding NPTII into the genome of the plants. Genomic DNA was isolated from leaves (approximately 15 cm. long) from approximately 4 month-old pineapple plants grown in light boxes according to the method of Doyle et al. [Doyle et al. (1990) Focus 12:13-15]. The final DNA pellet was dissolved in H_2O or TE buffer (10 mM Tris, 1 mM EDTA, pH 8) and quantified on a 0.7-1.0% agarose gel following electrophoresis. Isolated genomic DNA was digested with *EcoR*I and *Hind*III and hybridized to a probe obtained by digesting pBI426 with *EcoR*I and *Hind*III.

A modified alkaline transfer procedure [Meinkoth and Wahl (1984)] was performed for DNA Southern hybridization analysis [Southern (1975)] using a Gybond Plus nylon membrane (Amersham, Arlington Heights, Il). After an overnight blotting, the membrane was rinsed in 2xSSC buffer (1 x SSC=0.3 M NaCl, 0.03 M Na-citrate, pH 7.0) before drying for immobilization. DNA blots were first pre-hybridized at 65°C in 2.5 ml hybridization/100 cm^2 blot. Probes used in hybridization were either labeled with radioactive α-P^{32}-dCTP (Amersham) or non-radioactive digoxygenin-11-dUTP (DIG High Prime DNA Labeling and Detection Kit, Boehringer Mannheim). An equivalent of 1-2 x 10^7 cpm radioactive probe or 5-25 ng of non-radioactive probe was denatured for 10 min in a boiling water bath and immediately quenched in an ice/water bath before adding to the hybridization solution. Hybridized blots were washed for 5 min twice in 2 x SSC, 0.1% SDS at room temperature, followed by 15 min twice in 1.5xSSC, 0.1% SDS at 65°C each. Hybridized signals were then visualized by direct exposure to X-ray film (radioactive probed) or y chemiluminescence detection(non-radioactive probe) employing "CSPD" (Boehringer Mannheim). The results are shown in Figure 15.

Figure 15 shows a Southern hybridization blot of genomic DNA isolated from pineapple leaves transfected with pBI426 and PBI121 using biolistic bombardment; lane 1: 750 pg digested pBII21, equivalent to 10 copies of gene insertion into the pineapple genome; lane 2: 75 pg digested pBI121, equivalent to 1 copy of gene insertion; lane 3: 8 µg digested genomic DNA extracted from one transgenic pineapple plant line, transformed with pBI121 using particle bombardment; lane 4: 300 pg digested pBI426, equivalent to 10 copies of gene insertion; lane 5: 30 pg digested

pBI426, equivalent to 1 copy of gene insertion; lane 6: 8 μg digested genomic DNA extracted from an untransformed pineapple plant; lane 7: 8 μg digested genomic DNA extracted from a transgenic pineapple plant line, transformed with pBI426 particle bombardment. The results in Figure 15 demonstrate the integration of pBI121 (3.0 Kb band in lane 3) and of pBI426 (3.6 Kb band in lane 7) into the genome of pineapple leaves.

These data demonstrate the successful stable transformation of plbs. Furthermore, these data also demonstrate the successful generation of stably transformed transgenic pineapple plants from these transformed pineapple plbs. In particular, biolistic gene bombardment separately with each of the expression plasmids pBI426 and pBI121, and co-transformation with pAHC27 and pHA9 resulted in stably transformed pineapple plbs and regenerated pineapple plants.

In addition, stable transformation of plbs which were transformed by a combination of biolistic bombardment and *Agrobacterium* infection with the LBA4404 strain (Table 5) was also observed. Stably transformed plbs were selected for several months in G418-containing and showed high levels of NPTII protein as detected by ELISA. These results demonstrate the successful transformation of pineapple plbs by using a combination of biolistic bombardment and *Agrobacterium* infection using the liquid selection scheme as described above. This is in contrast to earlier experiments in which transformation with LBA4404 or LBA4301 was unsuccessful when selection on agar (rather than in liquid medium) in the absence of a recovery period (rather than 1-2 weeks for recovery in the absence of antibiotics) was used.

In contrast to the success in stably transforming pineapple plbs either by biolistic gene bombardment alone or by microwounding in combination with *Agrobacterium* infection, bombardment of exposed shoot apical meristems with pBI426 did not result in any stably transformed shoot meristems.

Also in contrast to the successful stable transformation of pineapple plbs with biolistic gene bombardment or with microwounding and *Agrobacterium* infection,

etiolated shoots which were inoculated with *Agrobacterium* were not successfully transformed.

EXAMPLE 3

Transformation of Pineapple Plbs With pROK CpTi+5 And Generation Of Nematode Resistant Pineapple Plants

Nematode control of the pineapple crop is a major problem for several reasons. Pineapple lacks natural resistance to nematodes. This necessitates the use of nematicides which are expensive and add to a grower's input costs. Additionally, even when nematicides are used to control nematode infestation of pineapple, nematodes can impose up to 40% loss of yield in the 30 to 36 month growing period of the crop that follows nematicide application. Furthermore, the two major fumigant pre-plant nematicides (*i.e.*, methyl bromide and 1,3-dichloropropene (Telone II)) are environmentally unacceptable; methyl bromide is a carcinogen, causes bromide build-up in soils, and is believed to be a primary cause of the depletion of the ozone layer; telone also causes halogen accumulation in soils and presents a major health risk for agricultural workers using this pesticide. Thus, there is a need for an environmentally acceptable solution to the nematode problem in pineapple.

Proteinase inhibitors, *e.g.*, trypsin inhibitors, are known to be potent anti-nematode agents (*see, e.g.*, Hepher *et al.*, U.S. Patent No. 5,494,813, the entire contents of which are herein incorporated by reference). In particular, transformation with the cowpea trypsin inhibitor (CpTi) has been shown to confer nematode resistance to potatoes (U.S. Patent No. 5,494,813, hereby incorporated by reference). Similarly, delivery of proteinase inhibitor genes to pineapples by transformation of pineapple plants with a DNA sequence encoding the CpTi could confer nematode resistance on transformed pineapple plants. This Example is divided into (a) generation of pineapple plants transgenic for CpTi, and (b) screening transgenic pineapple plants for nematode resistance.

A. Generation of pineapple plants transgenic for CpTi

The structure of plasmid pROK CpTi+5 which contains a nucleic acid sequence which encodes CpTi has been previously reported (U.S. Patent No. 5,494,813 hereby incorporated by reference). Transgenic pineapple plants are produced by bombarding pineapple plbs generated as described above (*see*, Example 1) with pROK CpTi+5 using a helium gas-driven apparatus (PDS-1000/He) (BioRad). DNA carriers, *e.g.*, tungsten (Sylvania M10, 1.1-μm-diameter) and gold (1.6-μm-diameter, BioRad) are used to precipitate DNA onto the mircroparticles according to the manufacturer's directions. Flight distance of the particles is 7.5 cm, target plbs are placed in 2.5-cm-diameter areas in the center of 10-cm-diameter petri dish with modified MS media containing 0.8% Difco Bacto agar and 3% sucrose. Bombardment with two shots is carried out at different helium pressures (*e.g.*, 1100 or 1550 psi). Controls are bombarded with uncoated particles.

Histochemical GUS staining is performed, as described *supra*, 48 hours following bombardment to determine transient transfection. Transformed plbs are selected by culturing for one month in 20-30 mg/ml G418. Transformed plbs, *i.e.*, plbs which survive the G418 selection, are used for the generation of plants as described in Example 1, and the regenerated G418-resistant plants screened for stable transformation by determining (a) NPTII expression as described *supra*, (b) expression of CpTi by Western blotting as described in U.S. Patent No. 5,494,813, or (c) Southern blotting to detect the CpTi coding sequence and/or *neo* coding sequence as described in U.S. Patent No. 5,494,813.

For Western blotting, soluble protein is extracted in PBS, electrophoresed on a 17% polyacrylamide gel, transferred to nitrocellulose and CpTi detected using rabbit anti-CpTi primary antibodies followed by horseradish peroxidase-conjugated donkey anti-rabbit secondary antibodies (Amersham) and autoradiography.

For Southern blotting, genomic DNA is extracted from different plant tissues or from the entire plant, digested with HindIII, and the digest separated on agarose gel, blotted onto filters and the filter-immobilized DNA hybridized with one of two labeled

probes (*i.e.*, a 564 bp AluI/ScaI fragment from pUSSR c3/2 which contains the CpTi coding sequence, and the 800 bp PstI/BamHI fragment from pNEO which contains the coding sequence for the NPTII gene). Since a unique HindIII restriction site is present between the CpTi and NPTII genes, a HindIII digest of genomic DNA containing a single copy of the T-DNA will generate two junction fragments. As the site of integration varies between different transformants, the size of the junction fragments also differs. Multiple copies of the T-DNA integrated at different sites within the same genome produces a commensurate number of bands when probed with a sequence specific to one of the junctions, thus allowing unambiguous determination of gene copy number.

B. Screening transgenic pineapple plants for nematode resistance

Nematode resistance of pineapple plants transgenic for the CpTi gene is determined by the infestation of transgenic and control wild-type plants with nematodes and the determination following different periods of infestation of a reduction in the number and size of nematodes, and in the proportion of female nematodes.

Pineapple plants of different ages (*i.e.*, newly planted, 1-, 2-, 3-, 4-, 5-, 6-, 7- and 8-month-old) are transferred from liquid culture to small pots with a diameter of 5 cm containing sand with grains of 50-400 μm to favor nematode invasion. A total of 500 freshly hatched *Rotylenchulus reniformis*, or *Meloidogyne javanica* nematodes are added to each pot. After four days, the sand and nematodes which failed to invade are washed from the plant, and the washed plant replanted in a sandy loam soil. The number, volume, and sex ratios of nematodes on the plant is assessed at different times (*e.g.*, 2, 4, 6, 8. 12 and 21 days) post-invasion using methods well known in the art. Wild-type plants generated from wild-type plbs, or from plbs bombarded with particles alone, are included as controls. A reduction in the number or volume of nematodes, or in the percentage of female nematodes on transgenic plants as compared with wild-type plants is expected. Such a reduction demonstrates that the transgenic pineapple plants are resistant to nematode invasion.

EXAMPLE 4

Transformation of Pineapple Plbs With Thaumatin Protein and Generation of Pineapple Fruit With High Sweetness

Harvesting of pineapple fruit is usually delayed in order to allow the production of sugars by the fruit and the development of a sweet flavor. However, delayed harvesting is undesirable since it shortens the fruit's shelf life and is associated with a translucency problem and pale color of the fruit which is not appealing to consumers. It is therefore desirable to express a sweet polypeptide in pineapple in order to allow earlier harvesting without sacrificing the flavor, appearance or shelf life of the fruit

Thaumatin is a protein originating from the arils of the fruit of *Tahumatococcus daiellii*. On a weight basis, thaumatin is 1600 times sweeter than sucrose. The nucleic acid sequence encoding thaumatin protein and constructs for expression of the protein in prokaryotes have been previously reported (U.S. Patent No. 4,891,316, the contents of which are herein incorporated by reference). Pineapple plbs are transformed with constructs which express the thaumatin gene. This results in the production of the sweet thaumatin protein and the generation of pineapple fruit of increased sweetness. The nucleic acid sequence which encodes thaumatin is inserted using methods well known in the art into the pBI426 plasmid described *supra* such that expression of the thaumatin nucleic acid sequence is under the control of the double 35S promoter sequence. Transformed plbs are selected for G418 resistance, and the G418-selected plbs allowed to regenerate pineapple plants. Transformed plants are allowed to produce pineapple fruit in the field, and the fruit is assessed for the expression of thaumatin and for sweetness. Stable transformation of thaumatin-encoding nucleic acid sequences in fruit is detected by Southern hybridization of genomic DNA from the fruit with probes specific for the thaumatin nucleic acid sequence. The presence of the thaumatin protein in fruit is detected by Western blot analysis using a monoclonal or polyclonal anti-thaumatin antibody generated using methods well known in the art. Additionally, sweetness is subjectively compared between wild-type fruit and fruit which are stably transformed with thaumatin nucleic acid sequences. It is expected that fruit transformed with thaumatin-encoding nucleic

acid sequences express thaumatin and have increased sweetness as compared with wild type fruit. The increased expression of thaumatin and/or of sweetness demonstrates expression of thaumatin protein in the transformed pineapple fruit.

EXAMPLE 5

Transformation of Pineapple Plbs With ACC Deaminase and Generation of Pineapple Fruit With Delayed Ripening

The short harvesting season and the short half life of pineapples as a result of the natural ripening process are a major problem in the industry which ultimately result in a higher cost of the fruit to the consumer. It is known that ethylene production stimulates pineapple flowering and fruit ripening. Ethylene is produced by the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene by the ethylene forming enzyme ACC oxidase. The expression of an ACC metabolizing enzyme (*e.g.*, ACC deaminase) in the desired plant tissue has been shown to reduce the level of ethylene in that tissue and to delay ripening. In this Example, pineapple fruit with an extended harvesting season and an extended shelf life are produced by transformation of pineapple plbs with DNA encoding ACC deaminase and regeneration of transformed pineapple plants from these plbs.

The nucleotide sequence of ACC deaminase and constructs (*e.g.*, pMON977) capable of expression of ACC deaminase in plants have been previously described (U.S. Patent No. 5,512,466 the contents of which are herein incorporated by reference). The pMON977 expression plasmid which is capable of expressing ACC deaminase protein under the transcriptional control of a CaMV35S promoter sequence, and which contains a NPTII selection gene sequence is used to transform pineapple plbs by biolistic bombardment as described above. Transformed plbs are screened for G418 resistance as described *supra*. Plbs which survive G418 selection are used for the generation of plants as described in Example 1, *supra*. Plants are transferred to the field to produce fruit. Stable transformation of the ACC deaminase gene into plbs, plants, and fruit is determined by screening for the presence of NPTII gene and measuring ACC deaminase activity in converting α-ketobutyrate acid to ethylene as

previously described (U.S. Patent No. 5,512,466). Plants which express the ACC deaminase gene are examined for inhibition of fruit ripening by scoring the timing when fruit begin to ripen, and the number of fruit entering the ripening stage. Wild-type pineapple plants are included as controls. Delay in the initiation of ripening and/or the time it takes to reach a fully ripe state is expected. Such delay demonstrates that expression of the AC deaminase gene delays pineapple fruit ripening.

EXAMPLE 6

Transformation of Pineapple Callus With ACC Oxidase Antisense Nucleic Acid Sequences and Generation of Pineapple Fruit With Delayed Ripening

In order to reduce ethylene production in pineapple, transgenic pineapple plants which express antisense RNA that is specific for mRNA which is encoded by pineapple ACC oxidase. Pineapple ACC oxidase sequences are isolated from a cDNA library is prepared from pineapple fruit tissue using methods known in the art and screened by hybridization with cDNA sequences derived from pTOM13. pTOM13 contains DNA which is believed to code for tomato ACC oxidase (Grierson *et al.*, U.S. Patent No. 5,365,015, the contents of which are herein incorporated by reference). Tomato plants which were transgenic for a DNA sequence that encoded RNA which was antisense to the tomato ACC oxidase mRNA showed at least 80% reduction in the level of ethylene present during fruit ripening (U.S. Patent No. 5,365,015).

This example involves (a) construction of a pineapple cDNA library, (b) cloning and characterization of pineapple fruit-specific ACC oxidase oligonucleotides homologous to tomato ACC oxidase cDNA, (c) construction of vectors for expression of anti-sense pineapple ACC oxidase transcripts, and (d) transformation of pineapple callus and regeneration of transgenic pineapple plants.

A. Construction of a Pineapple cDNA Library

Total RNA is isolated from fruit tissue of *Ananas comosus* (L.) cultivar (F153) using the method of Su and Gibor [Anal. Biochem. 174:650 (1988)] as modified by

Lopez-Gomez and Gomez-Lim [J. Plant Physiol. 141:82 (1992)]. Messenger RNA (polyA$^+$ RNA) is isolated using the PolyATtract® mRNA isolation system IV (Promega).

First and second strand cDNA is synthesized using the ZAP-cDNA synthesis kit (Stratagene). The double-stranded cDNA is recovered and blunt ended using Klenow enzyme (Stratagene). *Eco*RI linkers [5'-AATTCGGCACGAG-3' (SEQ ID NO:3) and 5'-CTCGTGCCG-3'] are added to the cDNA. *Xho*I "sticky ends" are generated at the end of the cDNA which corresponds to the 3' end of the mRNA by digestion of the *Xho*I site in the linker-primer. cDNA with *Eco*RI sticky ends at the 5' end and *Xho*I sticky ends at the 3' end (relative to the original mRNA) is electrophoresed on a 1% agarose gel to determine the size distribution of the cDNA. Fractions containing the largest cDNA molecules are ligated into Uni-ZAP™, a lambda ZAP vector digested with *Eco*RI and *Xho*I (Stratagene).

B. Cloning and Characterization of Pineapple Fruit-Specific ACC Oxidase Oligonucleotides Homologous To Tomato ACC Oxidase cDNA

Pineapple cDNA clones are probed with a 1.3 kb cDNA insert cut by PstI from pTOM13 (U.S. Patent No. 5,365,015). The 1.3 kb cDNA pTOM insert is radioactively labeled with Prime-a-Gene® random labeling system (Promega). Recombinant phages are screened by plaque-lifting onto Magna nylon or NitroPure nitrocellulose membranes (MSI Corp.) by fixing recombinant phage DNA to the membranes. The membranes are placed sequentially on filter paper saturated with 0.5 M NaOH and 1.5 M NaCl for five minutes, then on filter paper containing 0.5 M Tris-HCl (pH 8.0) and 1.5 M NaCl and finally on paper containing 2 X SSC and 0.2 M Tris-HCl for 30 seconds.

The DNA is cross linked to the nylon membranes using UV light (12,000 μJoules) using a UV Stratalinker 1800 (Stratagene) and baking at 80°C for 1 hour under vacuum. The membranes are prehybridized in 50 ml of 5X SSPE, 5X Denhardt's solution, 0.5% SDS and 100 μg/ml sonicated salmon sperm DNA for 1 hour. Hybridization is carried out in the same buffer containing the labelled probe at

65°C in a Hybaid Mark II hybridization oven. Clones which hybridize to the pineapple ACC oxidase probe are expected to be obtained. Clones are subjected to DNA sequencing using the Sequenase® version 2.0 DNA sequencing kit (USB) with either the T3 or T7 promoter primers.

C. Construction of Vectors for Expression of Anti-Sense Pineapple ACC Oxidase Transcripts

An expression vector containing pineapple ACC oxidase cDNA is constructed by releasing the cDNA from the lambda ZAP vector plasmid by restriction enzyme digestion and insertion into a pBI121 expression vector. The digestion products are separated by electrophoresis on a 1% SeaPlaque agarose gel, the cDNA is recovered and purified using the Genclean II kit (Bio101, Vista, CA) and inserted into pBI121 in-frame with the promoter. The resulting plasmid is electroporated into *E. coli* XL1-Blue cells (Stratagene) and transformants are expected to be obtained.

The presence and reverse orientation of the pineapple ACC oxidase cDNA sequences in pBI121 is confirmed using restriction mapping and DNA sequencing of the junctions between the plasmid and cDNA.

D. Transformation of Pineapple Callus and Regeneration of Transgenic Pineapple Plants

1. Culture conditions

Callus cultures from pineapple F153 crowns are imitated as described in Example 1, *supra*, for plb culture except that initiation is on agar-MS medium supplemented with 5.4 mg/L NAA, 5.2 mg/L IAA and 2.1 mg/L kinetin as described by Mathews et al (1981) Scientia Hor. 11:319-328. Once callus cultures are initiated, they are maintained on agar-MS medium with 400 mg/L casein hydrolysate and 10 mg/L NAA with 15% (v/v) coconut water.

2. Bombardment conditions

Pineapple calli are bombarded using helium gas-driven microprojectile accelerator (PDS 1000/He). Preparation of calli for bombardment is the same as that for plb preparation for bombardment as described in Example 2, *supra*. Bombardment conditions are determined by changing helium pressure between about 1100 psi and about 1500 psi, and the optimum conditions are used for callus bombardment in further experiments. Each plate of calli is bombarded with about 2 μg to about 6 μg of plasmid pBI121 which expresses antisense pineapple ACC oxidase.

3. Selection of transformed callus

Bombarded calli are selected on liquid or agar-MS medium with 400 mg/L casein hydrolysate and 10 mg/L NAA with 15% (v/v) coconut water containing the selection antibiotic G418 to select cells which express the NPTII gene. The amount of G418 used for selection of F153 callus is 20-30 mg/L.

Transformation of callus selected for neomycin resistance is confirmed by Southern analysis using pineapple ACC oxidase cDNA as probe. Transformed callus is used to regenerate pineapple plants. Fruit derived from transformed and from control callus (*i.e.*, unmodified pineapple or pineapple transformed with pBI121) are observed for signs of ripening. ACC oxidase mRNA expression and accumulation is measured by Northern blotting using pineapple ACC oxidase cDNA as probe. Ethylene production is measured in control and transgenic (*i.e.*, which express anti-sense sequences) pineapple fruit by enclosing individual fruits in sealed jars and detecting ethylene in the head space of the jar by gas chromatography. It is expected that fruit from transformed pineapple plants which express ACC oxidase antisense will produce a reduced level of ethylene, and will ripen more slowly, as compared to control pineapple fruit. Pineapple plants may be vegetatively propagated. Plants which are homozygous for the transgene may be produced from seeds and used for further breeding. Fruit produced by transgenic plants are expected to have the characteristics of delayed ripening (due to inhibition of endogenous ethylene

production). It is also expected that ripening will be inducible by exposure of the transgenic fruit to exogenous ethylene.

As clear from the above data, the present invention provides methods for producing pineapple-like plants with desirable characteristics. In particular, the invention provides methods for producing transgenic pineapple-like totipotent bodies which are useful in generating pineapple-like plants. Specifically, the present invention provides methods for producing transgenic pineapple-like totipotent bodies using biolistic bombardment alone or in combination with *Agrobacterium* transformation. Also provided by this invention are methods for culturing pineapple-like totipotent bodies which are useful in long-term culture and selection of transgenic totipotent bodies of pineapple-like plants.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and compositions of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art and in fields related thereto are intended to be within the scope of the following claims.